

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	3191	trypsin near6 chymotrypsin	USPAT	OR	OFF	2005/07/28 16:01
L3	208	(trypsin near6 (convert or conversion or converted)) or (chymotrypsin near6 (conversion or convert or converted))	USPAT	OR	OFF	2005/07/28 16:01
L4	840	chymotrypsin near4 (substrate or active or activity)	USPAT	OR	OFF	2005/07/28 16:01
L5	24	l1 and l3 and l4	USPAT	OR	OFF	2005/07/28 16:09
L6	72	trypsin near4 (mutation or mutant or mutated or variant)	USPAT	OR	OFF	2005/07/28 16:10
L7	7	L1 and l4 and l6	USPAT	OR	OFF	2005/07/28 16:10

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	3191	trypsin near6 chymotrypsin	USPAT	OR	OFF	2005/07/28 16:01
L3	208	(trypsin near6 (convert or conversion or converted)) or (chymotrypsin near6 (conversion or convert or converted))	USPAT	OR	OFF	2005/07/28 16:01
L4	840	chymotrypsin near4 (substrate or active or activity)	USPAT	OR	OFF	2005/07/28 16:01
L5	24	l1 and l3 and l4	USPAT	OR	OFF	2005/07/28 16:09
L6	72	trypsin near4 (mutation or mutant or mutated or variant)	USPAT	OR	OFF	2005/07/28 16:10
L7	7	L1 and l4 and l6	USPAT	OR	OFF	2005/07/28 16:10

Connecting via Winsock to STN

Welcome to STN International! Enter x:x

LOGINID:SSSPTA1623SQS

PASSWORD:

TERMINAL (ENTER 1, 2, 3, OR ?):2

* * * * * Welcome to STN International * * * * *
* *

NEWS	1		Web Page URLs for STN Seminar Schedule - N. America
NEWS	2		"Ask CAS" for self-help around the clock
NEWS	3	FEB 28	PATDPAFULL - New display fields provide for legal status
			data from INPADOC
NEWS	4	FEB 28	BABS - Current-awareness alerts (SDIs) available
NEWS	5	MAR 02	GBFULL: New full-text patent database on STN
NEWS	6	MAR 03	REGISTRY/ZREGISTRY - Sequence annotations enhanced
NEWS	7	MAR 03	MEDLINE file segment of TOXCENTER reloaded
NEWS	8	MAR 22	KOREAPAT now updated monthly; patent information enhanced
NEWS	9	MAR 22	Original IDE display format returns to REGISTRY/ZREGISTRY
NEWS	10	MAR 22	PATDPASPC - New patent database available
NEWS	11	MAR 22	REGISTRY/ZREGISTRY enhanced with experimental property tags
NEWS	12	APR 04	EPFULL enhanced with additional patent information and new fields
NEWS	13	APR 04	EMBASE - Database reloaded and enhanced
NEWS	14	APR 18	New CAS Information Use Policies available online
NEWS	15	APR 25	Patent searching, including current-awareness alerts (SDIs), based on application date in CA/CAPLUS and
USPATFULL/USPAT2			may be affected by a change in filing date for U.S. applications.
NEWS	16	APR 28	Improved searching of U.S. Patent Classifications for
			U.S. patent records in CA/CAPLUS
NEWS	17	MAY 23	GBFULL enhanced with patent drawing images
NEWS	18	MAY 23	REGISTRY has been enhanced with source information from
			CHEMCATS
NEWS	19	JUN 06	The Analysis Edition of STN Express with Discover!

(Version 8.0 for Windows) now available

NEWS 20 JUN 13 RUSSIAPAT: New full-text patent database on STN

NEWS 21 JUN 13 FRFULL enhanced with patent drawing images

NEWS 22 JUN 27 MARPAT displays enhanced with expanded G-group definitions

and text labels

NEWS 23 JUL 01 MEDICONF removed from STN

NEWS 24 JUL 07 STN Patent Forums to be held in July 2005

NEWS 25 JUL 13 SCISEARCH reloaded

NEWS 26 JUL 20 Powerful new interactive analysis and visualization software,

STN AnaVist, now available

NEWS EXPRESS JUNE 13 CURRENT WINDOWS VERSION IS V8.0, CURRENT MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP), AND CURRENT DISCOVER FILE IS DATED 13 JUNE 2005

NEWS HOURS STN Operating Hours Plus Help Desk Availability

NEWS INTER General Internet Information

NEWS LOGIN Welcome Banner and News Items

NEWS PHONE Direct Dial and Telecommunication Network Access to STN

NEWS WWW CAS World Wide Web Site (general information)

Enter NEWS followed by the item number or name to see news on that specific topic.

All use of STN is subject to the provisions of the STN Customer agreement. Please note that this agreement limits use to scientific research. Use for software development or design or implementation of commercial gateways or other similar uses is prohibited and may result in loss of user privileges and other penalties.

* * * * * STN Columbus * * * * *

FILE 'HOME' ENTERED AT 16:23:35 ON 28 JUL 2005

=> File Medline EMBASE Biosis Caplus

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.21	0.21

FILE 'MEDLINE' ENTERED AT 16:23:43 ON 28 JUL 2005

FILE 'EMBASE' ENTERED AT 16:23:43 ON 28 JUL 2005
 COPYRIGHT (C) 2005 Elsevier Inc. All rights reserved.

FILE 'BIOSIS' ENTERED AT 16:23:43 ON 28 JUL 2005
 Copyright (c) 2005 The Thomson Corporation

FILE 'CAPLUS' ENTERED AT 16:23:43 ON 28 JUL 2005
 USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.

PLEASE SEE "HELP USAGETERMS" FOR DETAILS.
COPYRIGHT (C) 2005 AMERICAN CHEMICAL SOCIETY (ACS)

=> s trypsin (6A) chymotrypsin
L1 22175 TRYPSIN (6A) CHYMOTRYPSIN

=> s (trypsin (6A) (convert or conversion or converted)) or
(chymotrypsin (6A) (conversion or convert or converted))
L2 1475 (TRYPSIN (6A) (CONVERT OR CONVERSION OR CONVERTED)) OR
(CHYMOTRY
PSIN (6A) (CONVERSION OR CONVERT OR CONVERTED))

=> s chymotrypsin (4A) (substrate or active or activity)
L3 10198 CHYMOTRYPSIN (4A) (SUBSTRATE OR ACTIVE OR ACTIVITY)

=> s trypsin (4A) (mutation or mutant or mutated or variant)
L4 1751 TRYPSIN (4A) (MUTATION OR MUTANT OR MUTATED OR
VARIANT)

=> s l1 and l2 and l3 and l4
L5 20 L1 AND L2 AND L3 AND L4

=> duplicate
ENTER REMOVE, IDENTIFY, ONLY, OR (?):remove
ENTER L# LIST OR (END):15
DUPLICATE PREFERENCE IS 'MEDLINE, EMBASE, BIOSIS, CAPLUS'
KEEP DUPLICATES FROM MORE THAN ONE FILE? Y/(N):n
PROCESSING COMPLETED FOR L5
L6 8 DUPLICATE REMOVE L5 (12 DUPLICATES REMOVED)

=> d l6 1-8 bib ab

L6 ANSWER 1 OF 8 MEDLINE on STN DUPLICATE 1
AN 2004155195 MEDLINE
DN PubMed ID: 15047908
TI Ala226 to Gly and Ser189 to Asp mutations **convert** rat
chymotrypsin B to a **trypsin**-like protease.
AU Jelinek Balazs; Antal Jozsef; Venekei Istvan; Graf Laszlo
CS Biotechnology Research Group of the Hungarian Academy of
Sciences.
SO Protein Eng Des Sel, (2004 Feb) 17 (2) 127-31. Electronic
Publication:
2004-01-20.
Journal code: 101186484. ISSN: 1741-0126.
CY England: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 200412
ED Entered STN: 20040330
Last Updated on STN: 20041222
Entered Medline: 20041221

AB In a previous successful attempt to **convert trypsin** to a **chymotrypsin**-like protease, 15 residues of **trypsin** were replaced with the corresponding ones in chymotrypsin. This suggests a complex mechanism of substrate recognition instead of a relatively simple one that only involves three sites, residues 189, 216 and 226. However, both **trypsin-->elastase** and **chymotrypsin-->trypsin conversion** experiments carried out according to the complex model resulted in non-specific proteases with low catalytic activity. Chymotrypsin used in the latter studies was of B-type, containing an Ala residue at position 226. Trypsins, however, contain a conserved Gly at this site. The substantially decreased trypsin-like activity of the G226A **trypsin mutant** also suggests a specific role for this site in substrate binding. Here we investigate the role of site 226 by introducing the A226G substitution into **chymotrypsin-->trypsin mutants** which were constructed according to both the simple (S189D mutant) and the complex model (S(1) mutant) of specificity determination. The kinetic parameters show that the A226G substitution in the S(1) mutant increased the **chymotrypsin-like activity**, while the **trypsin-like activity** did not change. In contrast, this substitution in the S189D chymotrypsin mutant resulted in a 100-fold increase in trypsin-like activity and a trypsin-like specificity profile as tested on a competing oligopeptide substrate library. Additionally, the S189D+A226G **mutant** is the first **trypsin-like chymotrypsin** that undergoes autoactivation, an exclusive property of trypsinogen among pancreatic serine proteases.

L6 ANSWER 2 OF 8 MEDLINE on STN DUPLICATE 2
AN 1998420367 MEDLINE
DN PubMed ID: 9749919
TI Converting trypsin to elastase: substitution of the S1 site and adjacent loops reconstitutes esterase specificity but not amidase activity.
AU Hung S H; Hedstrom L
CS Department of Biochemistry, Brandeis University, Waltham, MA 02254-9110, USA.
SO Protein engineering, (1998 Aug) 11 (8) 669-73.

Journal code: 8801484. ISSN: 0269-2139.

CY ENGLAND: United Kingdom

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199811

ED Entered STN: 19990115
 Last Updated on STN: 20000303
 Entered Medline: 19981130

AB The **conversion** of **trypsin** into a protease with **chymotrypsin-like activity** and specificity required substitution of fifteen residues in the S1 site and two surface loops with their chymotrypsin counterparts [Hedstrom, L., Szilagyi, L. and Rutter, W.J. (1992) Science, 255, 1249-1253]. These residues may define a set of general structural determinants of specificity in the trypsin family. In order to test this hypothesis, we have attempted to **convert trypsin** into a protease with specificity for substrates containing small aliphatic residues by replacing the S1 site and these surface loops with the analogous residues of elastase. Five elastase-like mutant enzymes were constructed with various combinations of these substitutions. Four mutant enzymes catalyze the hydrolysis of MeOSuc-Ala-Ala-Pro-Ala-SBzl more efficiently than the hydrolysis of Suc-Ala-Ala-Pro-Phe-SBzl. This observation indicates that the mutant enzymes have elastase-like esterase specificity. The best mutant, Tr-->E1-2, is a more specific esterase than elastase: the ratio of the values of kcat/Km for MeOSuc-Ala-Ala-Pro-Ala-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl is greater than 160 for Tr-->E1-2 and 50 for elastase. However, the esterase activity of Tr-->E1-2 is 300-fold less than elastase; in addition, Tr-->E1-2 has no measurable amidase activity. Thus these substitutions do not construct a protease with elastase-like activity. These experiments indicate that a unique structural solution is required for each different specificity. Previous work suggested that instability of the S1 site is a major barrier to redesigning the specificity of trypsin. This view is corroborated by

preliminary structural studies of Tr-->E1-2. One dimensional 1H NMR spectrum of Tr-->E1-2 suggests that the S1 site and the two surface loops of this **mutant trypsin** may be disordered.

L6 ANSWER 3 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN

AN 1997:481141 CAPLUS

DN 127:187447

TI Converting **trypsin** to **chymotrypsin**: Structural determinants of S1' specificity

AU Kurth, Torsten; Ullmann, Dirk; Jakubke, Hans-Dieter; Hedstrom, Lizbeth

CS Department of Biochemistry, Brandeis University, Waltham, MA, 02254, USA

SO Biochemistry (1997), 36(33), 10098-10104

CODEN: BICHAW; ISSN: 0006-2960

PB American Chemical Society

DT Journal

LA English

AB **Trypsin** and **chymotrypsin** differ strikingly in **substrate** specificities despite great similarity in their primary and tertiary structures. This work analyzes the role of 2 surface loops,

loop 40 and loop 60, as structural determinants of the specificity of the

S1'-subsite in **chymotrypsin** and **trypsin**.

Chymotrypsin prefers P1' Arg/Lys residues, whereas **trypsin** prefers hydrophobic P1' residues. Here, loops 40 and 60 were replaced in

trypsin with their **chymotrypsin** counterparts. These mutations did not affect the S1 specificity and catalytic activity of

trypsin. The S1' specificity was analyzed by monitoring acyl-transfer

reactions to 16 amino acid amides. The exchange of loop 40 did not affect

the S1' specificity. In contrast, the replacement of loop 60 caused a

loss of specificity for P1'-Met/Ile/Leu. Combining both mutations

reconstituted a **chymotrypsin**-like S1' specificity. The specificity for

Arg-NH2 increased 3-fold, whereas the preferences for Met-NH2 and Ile-NH2

decreased 4- and 8-fold, resp. Therefore, P1'-Arg/Met discrimination

changes by a factor of 12 and P1'-Arg/Ile discrimination changes by a

factor of 24. Thus, loop 40 and loop 60 act synergistically to determine S1'

specificity in **trypsin** and **chymotrypsin**.

RE.CNT 32 THERE ARE 32 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L6 ANSWER 4 OF 8 MEDLINE on STN
AN 96184403 MEDLINE
DN PubMed ID: 8612781
TI Attempts to **convert chymotrypsin to trypsin**.
CM Republished from: FEBS Lett. 1996 Jan 29;379(2):143-7. PubMed
ID: 8635580
AU Venekei I; Szilagyi L; Graf L; Rutter W J
CS University of California, San Francisco, Hormone Research
Institute, USA.
NC DK21344 (NIDDK)
SO FEBS letters, (1996 Mar 25) 383 (1-2) 143-7.
Journal code: 0155157. ISSN: 0014-5793.
CY Netherlands
DT (CORRECTED AND REPUBLISHED ARTICLE)
Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199606
ED Entered STN: 19960613
Last Updated on STN: 20010625
Entered Medline: 19960606
AB **Trypsin** and **chymotrypsin** have specificity pockets of
essentially the same geometry, yet **trypsin** is specific for basic
while **chymotrypsin** for bulky hydrophobic residues at the P1 site
of the substrate. A model by Steitz, Henderson and Blow
suggested the
presence of a negative charge at site 189 as the major
specificity
determinant: Asp189 results in tryptic, while the lack of it
chymotryptic
specificity. However, recent mutagenesis studies have shown
that a
successful **conversion** of the specificity of **trypsin** to
that of **chymotrypsin** requires the substitution of amino acids at
sites 138, 172 and at thirteen other positions in two surface
loops, that
do not directly contact the substrate. For further testing the
significance of these sites in **substrate** discrimination in
trypsin and **chymotrypsin**, we tried to change the
chymotrypsin specificity to **trypsin**-like specificity by
introducing reverse substitutions in rat chymotrypsin. We
report here
that the specificity conversion is poor: the Ser189Asp mutation
reduced
the **activity** but the specificity remained **chymotrypsin**
-like; on further substitutions the activity decreased further
on both
tryptic and chymotryptic substrates and the specificity was lost
or became

slightly trypsin-like. Our results indicate that in addition to structural elements already studied, further (chymotrypsin) specific sites have to be **mutated** to accomplish a **chymotrypsin --> trypsin specificity conversion**.

L6 ANSWER 5 OF 8 MEDLINE on STN
AN 96225958 MEDLINE
DN PubMed ID: 8635580
TI Attempts to **convert chymotrypsin to trypsin**.
CM Republished in: FEBS Lett. 1996 Mar 25;383(1-2):143-7. PubMed ID: 8612781
AU Venekei I; Szilagyi L; Graf L; Rutter W J
CS University of California, Hormone Research Institute, San Francisco, USA..
veheki@ludens.elte.hu
NC DK21344 (NIDDK)
SO FEBS letters, (1996 Jan 29) 379 (2) 143-7.
Journal code: 0155157. ISSN: 0014-5793.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199607
ED Entered STN: 19960719
Last Updated on STN: 20010625
Entered Medline: 19960705
AB **Trypsin** and **chymotrypsin** have specificity pockets of essentially the same geometry, yet **trypsin** is specific for basic while **chymotrypsin** for bulky hydrophobic residues at the P1 site of the substrate. A model by Steitz, Henderson and Blow suggested the presence of a negative charge at site 189 as the major specificity determinant: Asp189 results in tryptic, while the lack of it chymotryptic specificity. However, recent mutagenesis studies have shown that a successful **conversion** of the specificity of **trypsin** to that of **chymotrypsin** requires the substitution of amino acids at sites 138, 172 and at thirteen other positions in two surface loops, that do not directly contact the substrate. For further testing the significance of these sites in **substrate** discrimination in **trypsin** and **chymotrypsin**, we tried to change the **chymotrypsin** specificity to **trypsin**-like specificity by introducing reverse substitutions in rat chymotrypsin. We report here that the specificity conversion is poor: the Ser189Asp mutation reduced the **activity** but the specificity remained **chymotrypsin**-like; on further substitutions the activity decreased further on both

tryptic and chymotryptic substrates and the specificity was lost or became

slightly trypsin-like. Our results indicate that in addition to structural elements already studied, further (chymotrypsin) specific sites

have to be **mutated** to accomplish a **chymotrypsin--> trypsin specificity conversion**.

L6 ANSWER 6 OF 8 MEDLINE on STN DUPLICATE 3

AN 94312389 MEDLINE

DN PubMed ID: 8038166

TI Converting **trypsin** to **chymotrypsin**: ground-state binding does not determine substrate specificity.

AU Hedstrom L; Farr-Jones S; Kettner C A; Rutter W J

CS Graduate Department of Biochemistry, Brandeis University, Waltham,

Massachusetts 02254.

NC DK21344 (NIDDK)

SO Biochemistry, (1994 Jul 26) 33 (29) 8764-9.

Journal code: 0370623. ISSN: 0006-2960.

CY United States

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 199408

ED Entered STN: 19940905

Last Updated on STN: 19940905

Entered Medline: 19940825

AB Rat **trypsin** II has been **converted** to a protease with **chymotrypsin**-like **substrate** specificity [Hedstrom, L., et al. (1994) Biochemistry (preceding paper in this issue)].

The key

alteration in this conversion is the exchange of two surface loops for the

analogous loops of chymotrypsin. $k(\text{inact})/K_i$ for the inactivation of

chymotrypsin, **trypsin**, a **trypsin**

mutant with poor **activity** (D189S), and the

chymotrypsin-like mutants Tr-->Ch[S1+L1+L2] and

Tr-->Ch[S1+L1+L2+Y172W] by Suc-Ala-Ala-Pro-Phe-chloromethylketone correlates with k_{cat}/K_m for hydrolysis of

Suc-Ala-Ala-Pro-Phe-AMC.

$k(\text{inact})$'s for the inactivation of Tr-->Ch[S1+L1+L2] and

Tr-->Ch[S1+L1+L2+Y172W] are comparable to that of chymotrypsin,

while K_i 's

were much higher. K_i for the inhibition of these enzymes by the transition-state analog MeOSuc-Ala-Ala-Pro-boro-Phe also correlates with

k_{cat}/K_m for hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. These results suggest

that the surface loops stabilize the transition state for hydrolysis of

chymotrypsin substrates by improving the orientation of bound substrates relative to the catalytic residues. Lastly, **trypsin** and **chymotrypsin** have comparable affinities for proflavin, while the Kd for the Tr-->Ch[S1+L1+L2+Y172W]-proflavin complex is 10-fold higher. No proflavin binding could be observed for either D189S or Tr-->Ch-[S1+L1+L2], which suggests that the S1 binding pockets of these two mutant enzymes are deformed. This work confirms that enzyme specificity is expressed in the chemical steps of the reaction rather than in substrate binding.

L6 ANSWER 7 OF 8 MEDLINE on STN DUPLICATE 4
 AN 94312388 MEDLINE
 DN PubMed ID: 8038165
 TI Converting **trypsin** to **chymotrypsin**: residue 172 is a **substrate** specificity determinant.
 AU Hedstrom L; Perona J J; Rutter W J
 CS Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.
 NC DK21344 (NIDDK)
 GM13818-03 (NIGMS)
 SO Biochemistry, (1994 Jul 26) 33 (29) 8757-63.
 Journal code: 0370623. ISSN: 0006-2960.
 CY United States
 DT Journal; Article; (JOURNAL ARTICLE)
 LA English
 FS Priority Journals
 EM 199408
 ED Entered STN: 19940905
 Last Updated on STN: 19940905
 Entered Medline: 19940825
 AB **Trypsin** and **chymotrypsin** have very similar tertiary structures, yet very different substrate specificities. Recent site-directed mutagenesis studies have shown that mutation of the residues of the substrate binding pocket of **trypsin** to the analogous residues of **chymotrypsin** does not **convert trypsin** into a protease with **chymotrypsin**-like specificity. However, **chymotrypsin**-like **substrate** specificity is attained when two surface loops are changed to the analogous residues of chymotrypsin, in conjunction with the changes in the S1 binding site [Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) Science 255, 1249-1253). This mutant enzyme, Tr-->Ch[S1+L1+L2], is improved to a protease with 2-15% of the **activity** of

chymotrypsin by the mutation of Tyr172 to Trp. Residue 172 interacts synergistically with the residues of the substrate binding pocket and the loops to determine substrate specificity. Further, these **trypsin mutants** demonstrate that substrate specificity is determined by the rate of catalytic processing rather than by substrate binding.

L6 ANSWER 8 OF 8 CAPLUS COPYRIGHT 2005 ACS on STN
 AN 1992:422402 CAPLUS
 DN 117:22402
 TI Converting **trypsin** to **chymotrypsin**: the role of surface loops
 AU Hedstrom, Lizbeth; Szilagyi, Laszlo; Rutter, William J.
 CS Horm. Res. Inst., Univ. California, San Francisco, CA, 94143-0534, USA
 SO Science (Washington, DC, United States) (1992), 255(5049), 1249-53
 CODEN: SCIEAS; ISSN: 0036-8075
 DT Journal
 LA English
 AB **Trypsin** (Tr) and **chymotrypsin** (Ch) have similar tertiary structures, yet Tr cleaves peptides at arginine and lysine residues and Ch prefers large hydrophobic residues. Although replacement of the S1 binding site of Tr with the analogous residues of Ch is sufficient to transfer Ch specificity for ester hydrolysis, specificity for amide hydrolysis is not transferred. **Trypsin** is converted to a Ch-like protease when the binding pocket alterations are further modified by exchange of the Ch surface loops 185 through 188 and 221 through 225 for the analogous Tr loops. These loops are not structural components of either the S1 binding site or the extended substrate binding sites. This mutant enzyme is equivalent to Ch in its catalytic rate, but its substrate binding is impaired. Like Ch, this mutant utilizes extended substrate binding to accelerate catalysis, and substrate discrimination occurs during the acylation step rather than in substrate binding.

=> file stnguide

COST IN U.S. DOLLARS

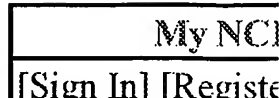
SINCE FILE

TOTAL

FULL ESTIMATED COST	ENTRY 45.53	SESSION .45.74
DISCOUNT AMOUNTS (FOR QUALIFYING ACCOUNTS)	SINCE FILE ENTRY	TOTAL SESSION
CA SUBSCRIBER PRICE	-1.46	-1.46

FILE 'STNGUIDE' ENTERED AT 16:25:25 ON 28 JUL 2005
 USE IS SUBJECT TO THE TERMS OF YOUR CUSTOMER AGREEMENT
 COPYRIGHT (C) 2005 AMERICAN CHEMICAL SOCIETY, JAPAN SCIENCE
 AND TECHNOLOGY CORPORATION, AND FACHINFORMATIONSZENTRUM KARLSRUHE

FILE CONTAINS CURRENT INFORMATION.
 LAST RELOADED: Jul 22, 2005 (20050722/UP).



All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Book

Search for

Display Show Sort by Send to

About Entrez

All: 1 Review: 0

Text Version

☐ 1: Biochemistry. 1994 Jul 26;33(29):8757-63. [Related Articles, Links](#)

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Database

Single Citation

Matcher

Batch Citation

Matcher

Clinical Queries

Special Queries

LinkOut

My NCBI

(Cubby)

Related

Resources

Order Documents

NLM Catalog

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

Converting trypsin to chymotrypsin: residue 172 is a substrate specificity determinant.

Hedstrom L, Perona JJ, Rutter WJ.

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.

Trypsin and chymotrypsin have very similar tertiary structures, yet very different substrate specificities. Recent site-directed mutagenesis studies have shown that mutation of the residues of the substrate binding pocket of trypsin to the analogous residues of chymotrypsin does not convert trypsin into a protease with chymotrypsin-like specificity. However, chymotrypsin-like substrate specificity is attained when two surface loops are changed to the analogous residues of chymotrypsin, in conjunction with the changes in the S1 binding site [Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) Science 255, 1249-1253]. This mutant enzyme, Tr-->Ch[S1+L1+L2], is improved to a protease with 2-15% of the activity of chymotrypsin by the mutation of Tyr172 to Trp. Residue 172 interacts synergistically with the residues of the substrate binding pocket and the loops to determine substrate specificity. Further, these trypsin mutants demonstrate that substrate specificity is determined by the rate of catalytic processing rather than by substrate binding.

PMID: 8038165 [PubMed - indexed for MEDLINE]

Display Show Sort by Send to

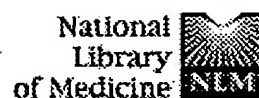
[Write to the Help Desk](#)

[NCBI](#) | [NLM](#) | [NIH](#)

[Department of Health & Human Services](#)

[Privacy Statement](#) | [Freedom of Information Act](#) | [Disclaimer](#)

Jul 26 2005 04:43:15



All Databases PubMed Nucleotide Protein Genome Structure OMIM PMC Journals Books

Search for

Limits Preview/Index History Clipboard Details

Display Show Sort by Send to

About Entrez

All: 1 Review: 0

Text Version

☐ 1: Biochemistry. 1994 Jul 26;33(29):8764-9. Related Articles, Links

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Database

Single Citation

Matcher

Batch Citation

Matcher

Clinical Queries

Special Queries

LinkOut

My NCBI

(Cubby)

Related

Resources

Order Documents

NLM Catalog

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

Converting trypsin to chymotrypsin: ground-state binding does not determine substrate specificity.

Hedstrom L, Farr-Jones S, Kettner CA, Rutter WJ.

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.

Rat trypsin II has been converted to a protease with chymotrypsin-like substrate specificity [Hedstrom, L., et al. (1994) Biochemistry (preceding paper in this issue)]. The key alteration in this conversion is the exchange of two surface loops for the analogous loops of chymotrypsin. $k(\text{inact})/K_i$ for the inactivation of chymotrypsin, trypsin, a trypsin mutant with poor activity (D189S), and the chymotrypsin-like mutants Tr-->Ch[S1+L1+L2] and Tr-->Ch[S1+L1+L2+Y172W] by Suc-Ala-Ala-Pro-Phe-chloromethylketone correlates with k_{cat}/K_m for hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. $k(\text{inact})$'s for the inactivation of Tr-->Ch[S1+L1+L2] and Tr-->Ch[S1+L1+L2+Y172W] are comparable to that of chymotrypsin, while K_i 's were much higher. K_i for the inhibition of these enzymes by the transition-state analog MeOSuc-Ala-Ala-Pro-boro-Phe also correlates with k_{cat}/K_m for hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC. These results suggest that the surface loops stabilize the transition state for hydrolysis of chymotrypsin substrates by improving the orientation of bound substrates relative to the catalytic residues. Lastly, trypsin and chymotrypsin have comparable affinities for proflavin, while the K_d for the Tr-->Ch[S1+L1+L2+Y172W]-proflavin complex is 10-fold higher. No proflavin binding could be observed for either D189S or Tr-->Ch-[S1+L1+L2], which suggests that the S1 binding pockets of these two mutant enzymes are deformed. This work confirms that

enzyme specificity is expressed in the chemical steps of the reaction rather than in substrate binding.

PMID: 8038166 [PubMed - indexed for MEDLINE]

Display Show Sort by Send to

[Write to the Help Desk](#)

[NCBI](#) | [NLM](#) | [NIH](#)

[Department of Health & Human Services](#)

[Privacy Statement](#) | [Freedom of Information Act](#) | [Disclaimer](#)

Jul 26 2005 04:43:15

Converting trypsin to elastase: substitution of the S1 site and adjacent loops reconstitutes esterase specificity but not amidase activity

Su-Hwi Hung¹ and Lizbeth Hedstrom²

Department of Biochemistry, Brandeis University, Waltham, MA 02254-9110, USA

¹Present address: Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL 32610, USA

²To whom correspondence should be addressed

The conversion of trypsin into a protease with chymotrypsin-like activity and specificity required substitution of fifteen residues in the S1 site and two surface loops with their chymotrypsin counterparts [Hedstrom, L., Szilagyi, L. and Rutter, W.J. (1992) *Science*, 255, 1249–1253]. These residues may define a set of general structural determinants of specificity in the trypsin family. In order to test this hypothesis, we have attempted to convert trypsin into a protease with specificity for substrates containing small aliphatic residues by replacing the S1 site and these surface loops with the analogous residues of elastase. Five elastase-like mutant enzymes were constructed with various combinations of these substitutions. Four mutant enzymes catalyze the hydrolysis of MeOSuc-Ala-Ala-Pro-Ala-SBzl more efficiently than the hydrolysis of Suc-Ala-Ala-Pro-Phe-SBzl. This observation indicates that the mutant enzymes have elastase-like esterase specificity. The best mutant, Tr→El-2, is a more specific esterase than elastase: the ratio of the values of k_{cat}/K_m for MeOSuc-Ala-Ala-Pro-Ala-SBzl and Suc-Ala-Ala-Pro-Phe-SBzl is greater than 160 for Tr→El-2 and 50 for elastase. However, the esterase activity of Tr→El-2 is 300-fold less than elastase; in addition, Tr→El-2 has no measurable amidase activity. Thus these substitutions do not construct a protease with elastase-like activity. These experiments indicate that a unique structural solution is required for each different specificity. Previous work suggested that instability of the S1 site is a major barrier to redesigning the specificity of trypsin. This view is corroborated by preliminary structural studies of Tr→El-2. One dimensional ¹H NMR spectrum of Tr→El-2 suggests that the S1 site and the two surface loops of this mutant trypsin may be disordered.

Keywords: substrate specificity/serine protease

Introduction

A major goal in protein engineering is to understand how structure determines function. The trypsin family of serine proteases is an ideal system to address this problem. The catalytic mechanism of these enzymes is well characterized and many X-ray crystal structures of the trypsin family are also available (Steitz *et al.*, 1969; Ruhlmann *et al.*, 1973; Stroud *et al.*, 1974; Sweet *et al.*, 1974; Bode *et al.*, 1989; Polgar, 1989). Trypsin, chymotrypsin and elastase have similar tertiary structures but very different substrate specificities. Trypsin is specific for substrates containing Lys/Arg in the P1 position; Asp189 is present at the bottom of the S1 site

(nomenclature of Schechter and Berger, 1967; chymotrypsinogen numbering). Chymotrypsin is specific for substrates containing Tyr/Phe/Trp in the P1 position; residue 189 is Ser in the S1 site of chymotrypsin. Elastase is specific for substrates containing Ala/Val in the P1 position; its S1 site is occluded by the side chains of Val216 and Thr226 (both residues are Gly in trypsin and chymotrypsin). These observations suggest that the specificity of enzymes in the trypsin family is determined by the residues at position 189, 216 and 226. However, the substitution of these residues in trypsin with analogous residues in chymotrypsin fails to transfer chymotrypsin-like activity to trypsin (Hedstrom *et al.*, 1992); the conversion of trypsin into a protease with chymotrypsin-like specificity requires substitution of the residues which comprise the S1 site and more distal structural elements (Hedstrom, 1996). Thus the specificity of trypsin is governed by a network of structural interactions.

The S1 sites of these enzymes are formed by residues 189–195, 214–220 and 225–228. Four S1 site residues differ between trypsin and chymotrypsin: 189, 192 (Gln in trypsin and Met in chymotrypsin), 217 (Tyr in trypsin and Ser in chymotrypsin) and 219 (absent in trypsin and Thr in chymotrypsin). In addition, Ser190 extends into the S1 site of trypsin but points out of the S1 site of chymotrypsin, where it forms a hydrogen bond with Thr138 (residue 138 is Ile in trypsin). Substitution of these residues is also not sufficient to convert trypsin into a protease with chymotrypsin-like specificity (Hedstrom *et al.*, 1992). However, trypsin is converted into a protease with chymotrypsin-like specificity when two surface loops are substituted with the corresponding residues of chymotrypsin in addition to the substitution of the residues comprising the S1 site (Hedstrom *et al.*, 1992). These loops connect the walls of the S1 site: loop 1 contains residues 185–188 and loop 2 contains residues 221–224. The mutant trypsin which contains all of these substitutions (Tr→Ch[S1+L1+L2]) has 0.1–1% of the activity of chymotrypsin (Hedstrom *et al.*, 1994). Chymotrypsin-like activity is further improved by the substitution of residue 172: Tyr172 forms hydrogen bonds to the carbonyl of Pro225 and the amide proton of Tyr217 in trypsin while Trp172 forms a single hydrogen bond to Pro225 in chymotrypsin. This trypsin mutant, Tr→Ch[S1+L1+L2+Y172W], has 10% of the activity of chymotrypsin. The structure of Tr→Ch[S1+L1+L2] resembles chymotrypsin, although loop 1 and much of loop 2 are disordered (Perona *et al.*, 1995). While the structure of Tr→Ch[S1+L1+L2+Y172W] displays significantly more order than in Tr→Ch[S1+L1+L2], disorder is still observed in loop 1. The instability of loop 1 may prevent Tr→Ch[S1+L1+L2+Y172W] from attaining the same activity as chymotrypsin. Importantly, both of these crystal structures utilized chloromethylketone inactivated enzymes. The observation of disorder even in the presence of an inhibitor suggests that loop 1 is even less structured in the unliganded enzyme; this disorder may even extend to loop 2 and the S1 site. The

structure of Tr→Ch[S1+L1+L2+Y172W] also suggested that loops 1 and 2 control the conformation of Gly216. The carbonyl of this residue forms a hydrogen bond to the NH of the P3 residue. Thus the conformation of Gly216 may be important in mediating the interactions of the S1 and S3 sites.

The residues of the S1 site, loops 1 and 2, and residue 172 define the set of structural elements required to change trypsin into a protease with chymotrypsin-like activity. In this paper, we test whether these structural elements are general determinants of specificity by replacing them with their elastase counterparts. The resulting enzymes have elastase-like esterase specificity, although they are not as active as elastase and do not possess measurable amidase activity. These results indicate that elastase specificity is determined by a different set of structural features, and suggests that each specificity requires a unique structural solution.

Materials and methods

Materials

Methoxy-succinyl-alanyl-alanyl-prolyl-alanine thiobenzylester (MeOSuc-AAPA-SBzl) was purchased from Enzyme Systems Products (Livermore, CA). Succinyl-alanyl-alanyl-prolyl-phenylalanine thiobenzylester (Suc-AAPF-SBzl), N-(carboxy) lysine thiobenzylester (Cbz-Lys-SBzl) and 4,4'-dithiodipyridine were purchased from Sigma Chemical Co. Acetyl-alanyl-alanyl-prolyl-alanine-7-amido-4-methylcoumarin (Ac-AAPA-AMC) were purchased from Bachem Bioscience Inc.

Construction of mutants

Mutants of rat trypsinogen II were constructed using the method of Kunkel as previously described (Kunkel, 1985; Hedstrom *et al.*, 1992). All mutants were completely sequenced to confirm that only the desired mutations are introduced.

Expression and purification of mutant trypsins

Recombinant rat trypsinogen II was produced as an α -factor fusion protein in a *Saccharomyces cerevisiae* expression system from the pYT plasmid, which contains the ADH/GAPDH promoter and α -factor leader sequence fused to the trypsinogen coding sequences (Hedstrom *et al.*, 1992). Mutant trypsinogens were purified from the culture media by cation exchange chromatography using a Toyopearl 650M column (Suppelco) with gradient of 100 mM AcOH/2 mM NaOAc (buffer A) to 100 mM Tris-HCl, pH 8.0 (buffer B). Trypsinogen was activated by addition of enterokinase and chromatographed on POROS HQ column (PerSeptive Biosystems) using the Biocad Sprint Perfusion Chromatography System. Trypsin mutants were stored in 1 mM HCl and 10 mM CaCl₂ at 4°C.

Activity of mutant trypsins

Hydrolysis of MeOSuc-AAPA-SBzl, Suc-AAPF-SBzl and Cbz-K-SBzl was monitored spectrophotometrically in the presence of 4,4'-dithiodipyridine ($\epsilon_{324} = 19.8 \text{ mM}^{-1} \text{ cm}^{-1}$) using a Hitachi U2000 spectrophotometer as previously described (Hedstrom *et al.*, 1994). Hydrolysis of Ac-AAPA-AMC was monitored fluorometrically, with excitation wavelength at 380 nm and emission wavelength at 460 nm using a PerSeptive Biosystems CytoFluor Multi-Well Reader, Series 400. Assay mix contained 10 mM CaCl₂, 0.1 M NaCl and 50 mM HEPES, pH 8.0. The kinetic data were analyzed using KinetAsyst software.

	138	172	182	185	190	195	216	220	226									
Rat trypsin II	i	y	c	v	g	f	l	e	g	k	d	s	c	g	d	s	g	s
Cow trypsin II	.	.	.	a	y
Rat chymotrypsin	t	w	.	a	.	a	s	v	s
Cow chymotrypsin A	t	w	.	a	.	a	s	v	s
Rat elastase I	i	w	.	a	.	g	d	.	v	r	s	g
Pig elastase I	i	w	.	a	.	g	d	.	v	r	s	g

Tr→Ch[S1+L1+L2+Y172W]	t	w
Tr→El-1	t	w
Tr→El-2	t	w
Tr→El-3	y	w
Tr→El-4	t	w
Tr→El-5	y	w

					loop 1											loop 2		

Fig. 1. Alignment of the S1 site and loops 1 and 2 in trypsin, chymotrypsin, elastase and trypsin mutants. Chymotrypsin numbering is used; (.) denote identity with rat trypsin II, and (-) denote gaps in the sequence. The residues of the S1 site are shown in capital letters. Loops 1 and 2 are identified by the dashed lines. Underlined lettering marks the changes between a mutant enzyme and its parent.

NMR spectroscopy of mutant trypsins

One dimensional ¹H-NMR spectra of mutant trypsins were recorded on a Bruker AMX-500 spectrometer. Samples contained Tr→El-2 or Ser195Ala (0.5 and 1.0 mM respectively) in 10% D₂O, 10 mM CaCl₂ and 10 mM deuterated Tris-HCl, pH 8.0. The activity of Tr→El-2 is stable over the course of measurement. However, wild-type trypsin degraded under these experimental conditions; therefore an inactive mutant, Ser195Ala, was used as a wild-type reference. The data were processed using the program Felix 95 (Biosym/MSI, CA).

Results and discussion

Construction, expression and purification of mutant trypsins

Five mutants of rat trypsin II were constructed with the goal of converting trypsin into a protease with elastase-like specificity (Figure 1). These mutants contained different combinations of substitutions at the S1 site, residues 138 and 172 and loops 1 and 2. Tr→Ch[S1+L1+L2+Y172W] was used as the parent because it already contained several of the required mutations (e.g., Asp189Ser, Tyr217Ser and Tyr172Trp). Tr→El-1 is designed to test the role of loops 1 and 2 by replacing the chymotrypsin-like loops of Tr→Ch[S1+L1+L2+Y172W] with the analogous sequences of elastase (Figure 1). Tr→El-2 is designed to imitate the occluded S1 site of elastase with the addition of Gly216Val and Gly226Thr to the modifications of Tr→El-1 (Figure 1). The S1 site of Tr→El-2 was further modified to produce Tr→El-3, Tr→El-4 and Tr→El-5. Tr→El-5 contains elastase-like substitutions which correspond to all of the modifications required to convert trypsin into an enzyme with chymotrypsin-like activity. If these residues define the structural determinants of specificity in the trypsin family of serine proteases, Tr→El-5 will possess elastase-like specificity.

All of the mutant enzymes were produced as zymogens in a *Saccharomyces cerevisiae* expression system and activated by enterokinase as described previously (Hedstrom *et al.*, 1994). Unlike previous mutants, the elastase-like enzymes do not bind to SBTI resin. Therefore, the elastase-like enzymes were purified using anion exchange chromatography. Enzyme concentration was determined by titration with *p*-nitrophenyl *p*'-guanidinobenzoate. The mutant enzymes were stable in 1 mM HCl, 10 mM CaCl₂, and did not autodegrade.

Criteria for characterization of elastase-like activity

Amide hydrolysis is generally more demanding, and more specific, than ester hydrolysis (Hedstrom *et al.*, 1992). For

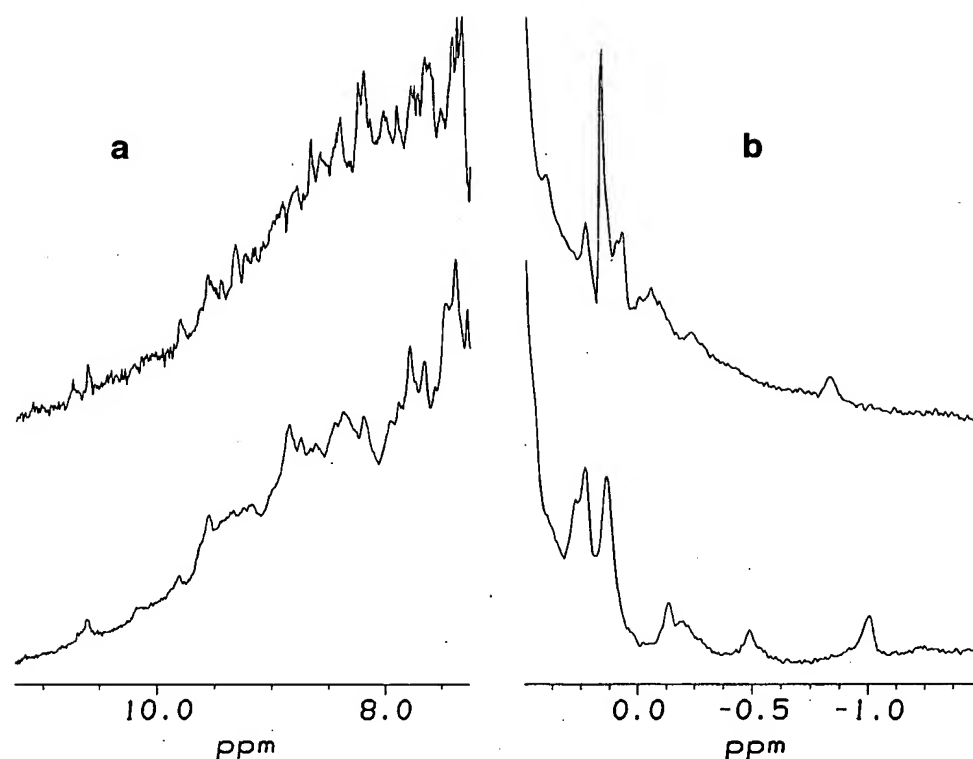


Fig. 2. One-dimensional ^1H NMR spectra (500 MHz) of rat trypsin and Tr→El-2. The Ser195Ala mutant of trypsin was used in these experiments to avoid autodegradation. The sample contained 0.9 mM enzyme in 10% D_2O , 10 mM CaCl_2 and 10 mM Tris, pH 8.0. The sample of Tr→El-2 contained 0.5 mM of enzyme in 10% D_2O , 10 mM deuterated Tris, 10 mM CaCl_2 , pH 8.0. Water suppression was achieved with 10^{-1} s presaturation pulse. Sweep width was 7042 Hz. FID's (512 scans and 4096 data points) were obtained at temperature of 25°C. (a) Top panel is Tr→El-2 at 7.6–11.2 p.p.m. range and bottom panel is trypsin at the same range. (b) Top panel is Tr→El-2 at 0.5 to –1.5 p.p.m. range and bottom panel is trypsin at the same range.

Table 1. Specificity of trypsin, elastase and elastase-like mutants

Enzyme	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)				
	MeoSuc-AAPA-SBzl	Suc-AAPF-SBzl	Ala:Phe	Cbz-K-SBzl	Ac-AAPA-AMC
Elastase	9×10^{5b} [1]	1.8×10^4 [1]	1 : 0.02	1.5×10^{4c} [1]	3.8×10^4 [1]
Trypsin	7×10^{3b} [0.01]	7.0×10^4 [4]	1 : 10	1.4×10^7 [10^3]	2.5×10^{-2b} [$<10^{-6}$]
Chymotrypsin	6.7×10^5 [0.7]	3×10^{6a} [40]	1 : 4	9×10^{3b} [0.6]	1.2^c [0.00003]
Tr→Ch[S1+L1+L2+Y172W]	1.5×10^5 [0.2]	6.0×10^6 [100]	1 : 40	1.0×10^4 [0.7]	18 ± 3 [0.00005]
Tr→El-1	2.2×10^4 [0.02]	1.2×10^{5b} [7]	1 : 5	2.2×10^2 [0.01]	$<10^{-2}$ [$<10^{-6}$]
Tr→El-2	3.1×10^3 [0.003]	<20 [$<10^{-3}$]	1 : <0.006	$<$ [<0.0003]	$<10^{-2}$ [$<10^{-6}$]
Tr→El-3	2.6×10^2 [3×10^{-4}]	<20 [$<10^{-3}$]	1 : <0.08	<1 [$<10^{-3}$]	n.d.
Tr→El-4	2.5×10^2 [3×10^{-4}]	<8 [<0.001]	1 : <0.03	<90 [<0.006]	n.d.
Tr→El-5	4.5×10^{2b} [5×10^{-3}]	<40 [<0.0002]	1 : <0.09	<20 [$<10^{-4}$]	n.d.

Conditions as described in Materials and methods. The brackets contain the activity relative to elastase. Ala:Phe, ratio of the value of k_{cat}/K_m for the hydrolysis of MeoSuc-AAPA-SBzl to that of Suc-AAPF-SBzl. n.d., no data. Error is less than 10% unless otherwise noted.

^aData from Hedstrom *et al.*, 1992. ^b30% error. ^c50% error.

example, the transfer of chymotrypsin-like esterase activity into trypsin requires only the substitution of Asp189 with Ser; more extensive substitutions do not alter esterase activity (Hedstrom *et al.*, 1992, 1994). In contrast, the transfer of chymotrypsin-like amidase activity requires fifteen substitutions. Therefore two substrates were chosen to assess elastase-like specificity: an amide, Ac-AAPA-AMC, and an ester, MeoSuc-AAPA-SBzl (Table I).

Only elastase efficiently hydrolyzed Ac-AAPA-AMC (Table I). Therefore the hydrolysis of Ac-AAPA-AMC can be used to monitor the transfer of elastase-like amidase activity.

However, the hydrolysis of Ac-AAPA-AMC by elastase is 10-fold less efficient than the hydrolysis of Suc-AAPF-AMC by chymotrypsin as measured by k_{cat}/K_m . This observation indicates that it will be more difficult to detect elastase-like activity in the mutant enzymes than to detect chymotrypsin-like activity in our previous experiments (Hedstrom *et al.*, 1994).

In contrast, the value of k_{cat}/K_m for the hydrolysis of MeoSuc-AAPA-SBzl by elastase is similar to that for hydrolysis by chymotrypsin, and only 100-fold greater than that by trypsin. These observations indicate that the increase in k_{cat}/K_m for the hydrolysis of MeoSuc-AAPA-SBzl will not be

a reliable measure of elastase-like activity. This lack of discrimination among these enzymes probably results from the high reactivity of the thiobenzyl ester and the ability of the P1 Ala residue to bind in the larger S1 sites of chymotrypsin and trypsin. This hypothesis is substantiated by the monitoring the hydrolysis of Suc-AAPF-SBzl and Cbz-K-SBzl. While these compounds are very good substrates of chymotrypsin and trypsin respectively, they are rather poor substrates of elastase. The value of k_{cat}/K_m for the hydrolysis of MeOSuc-AAPA-SBzl by elastase is 50-fold greater than those for the hydrolysis of Suc-AAPF-SBzl and Z-Lys-SBzl. This discrimination probably results from the inability of the smaller S1 site of elastase to accommodate P1 Phe and Lys residues. These observations suggest that elastase-like specificity may be assessed by monitoring the ratios of k_{cat}/K_m for the hydrolysis of MeOSuc-AAPA-SBzl and Suc-AABF-SBzl.

Elastase-like esterase specificity can be transferred to trypsin

Since esterase activity is more easily transferred than amidase activity, the ability of the five elastase-like mutant enzymes to hydrolyze MeOSuc-AAPA-SBzl, Suc-AAPF-SBzl and Cbz-K-SBzl was assessed first. The values of k_{cat}/K_m for the hydrolysis of MeOSuc-AAPA-SBzl are at least 300-fold less than that of elastase. Thus, we have not been able to construct a trypsin mutant with esterase activity comparable to elastase.

Tr→El-1 has the highest k_{cat}/K_m for the hydrolysis of MeOSuc-AAPA-SBzl. This enzyme also efficiently hydrolyzes Suc-AAPF-SBzl and Cbz-K-SBzl, although the values of k_{cat}/K_m for these substrates are 50-fold less than the parent enzyme Tr→Ch[S1+L1+L2+Y172W]. Thus Tr→El-1 is a non-specific esterase. This result is expected since Tr→El-1 does not contain the substitutions which occlude the S1 site. Nevertheless, the ratio of k_{cat}/K_m for the Ala and Phe changes from 1:40 for the parent enzyme to 1:5 for Tr→El-1. These observations indicate that substitution of loops 1 and 2 with their elastase counterparts selectively decreases the hydrolysis of substrates containing P1-Phe relative to P1-Ala residues.

The presence of the Gly216Val and Gly226Thr mutations should occlude the S1 site of Tr→El-2, further discriminating against substrates containing P1-Phe. Table I shows that this result is observed. The value of k_{cat}/K_m for the hydrolysis of MeOSuc-AAPA-SBzl by Tr→El-2 is 8-fold less than by Tr→El-1, while the value of k_{cat}/K_m for the hydrolysis of Suc-AAPF-SBzl decreases by 6000-fold. Thus the esterase specificity of Tr→El-1 is similar to that of elastase. These results demonstrate that the substitutions contained in Tr→El-2, i.e., loops 1 and 2, Gly216Val, Gly226Thr, Ile138Thr, Tyr172Trp, Gln192Met, Tyr217Ser and the insertion of Thr at position 219, are sufficient to convert trypsin into an enzyme with elastase-like esterase specificity, although not sufficient to reconstitute the activity of elastase.

The Ile138Thr, Gln192Met, and insertion of Thr at 219 substitutions in Tr→El-2 are carried over from the parent enzyme Tr→Ch[S1+L1+L2+Y172W]. It seemed likely that substitution of these residues with their elastase counterparts, Gln192Asn, Ile138Val and Gly at 219, might improve the mutant enzymes. In addition, the wall of the S1 site of elastase formed by residues 214 to 220 contains two extra residues and little sequence similarity to trypsin. Substitutions in this segment, in addition to the Gly216Thr and the insertion of Gly at 219 mutations already mentioned, might also improve the elastase-like specificity of the mutant enzymes. Tr→El-3, Tr→El-4 and Tr→El-5 were constructed within this objective.

The values of k_{cat}/K_m for hydrolysis MeOSuc-AAPA-SBzl by these mutants are 7- to 12-fold lower than that for hydrolysis by Tr→El-2. All of these mutants retained the discrimination between P1 Ala and Phe residues as judged by the ratios of k_{cat}/K_m . Therefore none of these mutant enzymes are an improvement over Tr→El-2 although their primary structure more closely resembles elastase. In particular, Tr→El-5 contains substitutions of all the structural elements which were required to transfer chymotrypsin-like activity and specificity into trypsin, yet it is less elastase-like than Tr→El-2.

In addition to the S1 site, the S3 site has been shown to play an important role in the specificity of elastase (Stein *et al.*, 1987). The acylation rate of human leukocyte elastase (HLE) increases 100-fold upon addition of a P3 residue. This observation suggests that the defect in the elastase-like mutants might result from the absence of proper P3-S3 interactions. However, the S3 site of elastase is comprised of residues 192, 216 and 217A (Bode *et al.*, 1989); these residues are also part of the S1 site, and were included in the substitution of Tr→El-5. Therefore the failure to observe elastase-like activity in the mutant enzymes does not result from the incorrect substitutions at the S3 site.

Elastase-like amidase activity is not reconstituted into the mutant enzymes

Amidase activity has more stringent structural requirements than esterase activity. Since the mutant enzymes have no more than 2.5% of the esterase activity of elastase, it was unlikely that they would have any elastase-like amidase activity. As expected, neither Tr→El-1 nor Tr→El-2 catalyze the hydrolysis of Ac-AAPA-AMC; the amidase activity of the remaining enzymes was not assayed since they have even less esterase activity than Tr→El-1 and Tr→El-2.

The substitutions may perturb the structure of the activation domain of trypsin

We measured the one dimensional ¹H-NMR spectra of trypsin and Tr→El-2 in order to qualitatively probe the effects of the mutations on enzyme structure. Both spectra are characterized by dispersed amide protons (7.6–11.2 p.p.m.) and methyl protons (0.5–1.5 p.p.m.) characteristic of folded protein structures (Figure 2). This observation suggests the substitutions do not alter the global structure of the trypsin. However, several of the upfield methyl protons resonances of the trypsin spectrum have shifted downfield or disappeared in the spectrum of Tr→El-2.

Previous studies of the ¹H-NMR spectra of bovine trypsin and trypsinogen observed a similar shift in the upfield proton resonances of trypsinogen relative to trypsin (Perkins and Wuthrich, 1980). The structures of trypsin and trypsinogen differ in the activation domain, which consists of residues 8–19, 142–153, 184A–193 and 216–223, and includes the S1 site, and loops 1 and 2. The activation domain is disordered in trypsinogen, and rigid in trypsin. Therefore, disorder in the S1 site and loops 1 and 2 correlate with shifts in the upfield proton spectra. These observations may suggest that the S1 site and loops 1 and 2 are also disordered in Tr→El-2.

Closer examination of these resonances further supports this view. The two highest field resonances of bovine trypsin were tentatively assigned to Ile73 (–1.0 p.p.m.) and Val227 (–0.8 p.p.m.). Both of these residues are conserved in rat trypsin, as are the neighboring residues, which suggests that similar resonances will be observed in the spectrum of rat trypsin (Figure 4b, bottom panel). Therefore it is likely that the two

highest field resonances at -1.0 and -0.45 p.p.m.) in the rat trypsin spectrum can also be assigned to Ile73 and Val227. Only one such high field resonance (-0.75 p.p.m.) is observed in Tr→El-2; this resonance could be either Ile73 or Val227. Tyr172 is adjacent to Val227; the Tyr172Trp substitution in Tr→El-2 is expected to change the shift of this upfield methyl resonance. However, none of the substitutions in Tr→El-2 are in the immediate vicinity of Ile73. Therefore changes in this methyl resonance suggest a more extensive structural perturbation, which is consistent with perturbation of the activation domain of Tr→El-2. These observations suggest that the failure to reconstitute elastase-like activity, as well as the remaining barrier in the conversion of trypsin into chymotrypsin, may result from disorder in loops 1 and 2 and the S1 site.

Conclusions

These observations suggest that the structural features which determine elastase specificity are different from those which confer chymotrypsin specificity. Similarly, Venekei *et al.* (1996) have shown that the analogous substitutions do not convert chymotrypsin into a protease with trypsin-like specificity. These studies suggest that different structural frameworks are required for different substrate specificities.

Acknowledgements

This work is supported by the grant of NSF Career MCB-9506805 and the Lucille P. Markey Charitable Trust to Brandeis University. L.H. is a Searle Scholar and a Beckman Foundation Young Investigator. The authors thank Dr Cathy Moore for the assistance with NMR spectroscopy and Annette Pasternak for the critical reading of the manuscript.

References

- Bode, W., Meyer, E.J. and Powers, J.C. (1989) *Biochemistry*, **28**, 1951–1963.
- Hedstrom, L. (1996) *Biol. Chem.*, **377**, 465–470.
- Hedstrom, L., Szilagyi, L. and Rutter, W.J. (1992) *Science*, **255**, 1249–1253.
- Hedstrom, L., Perona, J. and Rutter, W.J. (1994) *Biochemistry*, **33**, 8757–8763.
- Kunkel, T.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
- Perkins, S.J. and Wuthrich, K. (1980) *J. Mol. Biol.*, **138**, 43–64.
- Perona, J.J., Hedstrom, L., Rutter, W.J. and Fletterick, R.J. (1995) *Biochemistry*, **34**, 1489–1499.
- Polgar, L. (1989) *Mechanisms of Protease Action*. CRC Press, Inc., Boca Raton.
- Ruhlmann, A., Kukla, D., Schwager, P., Bartels, K. and Huber, R. (1973) *J. Mol. Biol.*, **77**, 417–436.
- Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 157–162.
- Stein, R.L., Strimpler, A.M., Hori, H. and Powers, J.C. (1987) *Biochemistry*, **26**, 1301–1305.
- Steitz, T.A., Henderson, R. and Blow, D.M. (1969) *J. Mol. Biol.*, **46**, 337–348.
- Stroud, R.M., Kay, L.M. and Dickerson, R.E. (1974) *J. Mol. Biol.*, **83**, 185–208.
- Sweet, R.M., Wright, H.T., Janin, J., Chothia, C.H. and Blow, D.M. (1974) *Biochemistry*, **13**, 4212–4228.
- Venekei, I., Szilagyi, L., Graf, L. and Rutter, W.J. (1996) *FEBS Lett.*, **379**, 143–147.

Received January 5, 1998; revised March 20, 1998; accepted April 10, 1998

Converting Trypsin to Chymotrypsin: Structural Determinants of S1' Specificity[†]

Torsten Kurth,[‡] Dirk Ullmann,^{‡,§} Hans-Dieter Jakubke,[§] and Lizbeth Hedstrom^{*,‡}

Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254, and Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Leipzig, Germany 04103

Received April 22, 1997; Revised Manuscript Received June 20, 1997[®]

ABSTRACT: Trypsin and chymotrypsin differ strikingly in substrate specificities despite great similarity in their primary and tertiary structures. This work analyzes the role of two surface loops, loop 40 and loop 60, as structural determinants of the specificity of the S1'-subsite in chymotrypsin and trypsin. Chymotrypsin prefers P1' Arg/Lys residues, while trypsin prefers hydrophobic P1' residues. We replaced loop 40 and loop 60 in trypsin with their chymotrypsin counterparts. These mutations do not affect the S1 specificity and catalytic activity of trypsin. The S1' specificity was analyzed by monitoring acyl-transfer reactions to 16 amino acid amides. The exchange of loop 40 does not affect the S1' specificity. In contrast, the replacement of loop 60 causes a loss of specificity for P1'-Met/Ile/Leu. Combining both mutations reconstitutes a chymotrypsin-like S1' specificity. The specificity for Arg-NH₂ increases 3-fold while the preferences for Met-NH₂ and Ile-NH₂ decrease 4- and 8-fold, respectively. Therefore, P1'-Arg/Met discrimination changes by factor 12 and P1'-Arg/Ile discrimination changes by factor 24. Thus, loop 40 and loop 60 act synergistically to determine S1' specificity in trypsin and chymotrypsin.

A major goal of biochemistry is to understand the structural basis of enzyme specificity. The trypsin family of serine proteases is an ideal model system for understanding this problem. Serine proteases are involved in many important physiological processes, including digestion, blood coagulation and fibrinolysis. Thus, they have been studied for decades and are among the best characterized classes of enzymes. The catalytic mechanism of serine proteases is well understood and dozens of X-ray crystal structures are available (Sweet et al., 1974; Stroud et al., 1974; Bode & Schwager, 1975; Huber et al., 1974; Polgar, 1989). Although trypsin and chymotrypsin have a very similar tertiary structures, they differ strikingly in their substrate specificities. Trypsin requires Lys/Arg residues in P1 while chymotrypsin has a strong preference for Phe/Tyr/Trp in P1 [nomenclature from Schechter & Berger (1967), Figure 1). Recently, chymotrypsin-like specificity was transferred into the rat trypsin II framework, thus identifying the structural determinants of S1 specificity (Hedstrom et al., 1992; Hedstrom et al., 1994; Perona et al., 1995). Surprisingly, the reconstitution of chymotrypsin-like activity required the replacement of two surface loops by the analogous loops of chymotrypsin (loop 1 residues 185–188, loop 2 residues 221–225) in addition to the substitutions in the S1 binding pocket (D189S, Q192M, I138T, insert T219) (Figure 2). These loops do not directly contact the substrates, but connect

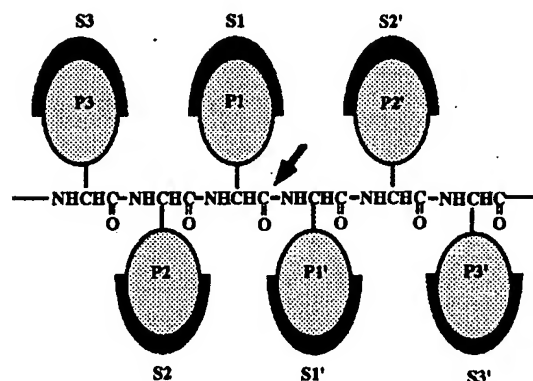


FIGURE 1: The subsites of proteases according to the nomenclature of Schechter and Berger (1967).

the walls of the S1 site. This enzyme, Tr→Ch[S1+L1+L2],¹ has 1% of the amidase activity of chymotrypsin. The chymotrypsin-like activity is further improved with an additional mutation, Y172W, which is also located outside the S1-site. This mutant, Tr→Ch[S1+L1+L2+Y172W], exhibits 15% of the chymotrypsin activity against amide substrates (Hedstrom et al., 1994). These mutant enzymes do not bind hydrophobic substrates well, but once bound, substrates are processed almost as efficiently as when bound to chymotrypsin. This observation illustrates the importance of the catalytic step of an enzymatic reaction for substrate discrimination.

Although the substrate specificity of trypsin and chymotrypsin is primarily determined by the S1 site, the other subsites also contribute. In particular, S' site interactions play a crucial role in the design of highly specific proteases and protease inhibitors and the use of proteases as peptide ligases (Bastos et al., 1995; Willett et al., 1995; Schellenberger & Jakubke, 1991). The S' subsite specificity has been determined for a number of proteases and the maximal

[†] This work was supported by the following organizations: German Scholarship Foundation (Studienstiftung des deutschen Volkes) (T.K.); German Academic Exchange Service (DAAD) (D.U.); NSF Career Award (L.H.); and a grant from the Lucille P. Markey Charitable Trust to Brandeis University. L.H. is a Searle Scholar and a Beckman Young Investigator. This work is Publication 1812 from the Department of Biochemistry, Brandeis University.

* Author to whom correspondence should be addressed.

[‡] Department of Biochemistry, Brandeis University, 415 South Street, Waltham Massachusetts 02254

[§] Institut fuer Biochemie, Fakultät fuer Biowissenschaften, Pharmazie und Psychologie, Universität Leipzig, Talstrasse 33, Leipzig, FRG 04103.

[®] Abstract published in *Advance ACS Abstracts*, August 1, 1997.

¹ Abbreviations: Tr, trypsin; Ch, chymotrypsin; AMC, Amino-methylcoumarin.

	S1' site				S1 site			
	33	40	58	66	138	172	185	192 216 225
Rat trypsin II	ln--sgyh		c-ykariqv		l	y	fleggkdsdq	gyg-calpdn
Cow trypsin	..-.-.		..-.-.		.	.	y..-.-.	..-.-.
Rat chymotrypsin A	..-.-.		..-.-.	-.-.	..-.-.
Rat chymotrypsin A	..-.-.		..-.-.	-.-.	..-.-.
Cow chymotrypsin A	..-.-.		..-.-.	-.-.	..-.-.
Cow chymotrypsin B	..-.-.		..-.-.	-.-.	..-.-.
Tr'→Ch' [L40]	..-.-.		..-.-.	-.-.	..-.-.
Tr'→Ch' [L60]	..-.-.		..-.-.	-.-.	..-.-.
Tr'→Ch' [L40+L60]	..-.-.		..-.-.	-.-.	..-.-.
Tr→Ch[S1+L1+L2+Y172W]	..-.-.		..-.-.		t	v	..-.-.	..-.-.
Tr→Ch[S1+L1+L2+Y172W+L40+L60]	..-.-.		..-.-.		t	v	..-.-.	..-.-.
	Loop 40				Loop 60			
					Loop 1			
					Loop 2			

FIGURE 2: Alignment of trypsin, chymotrypsin, and mutant trypsins. Chymotrypsinogen numbering is used. Periods denote residues which are identical to rat trypsin II, while (—) denotes gaps in the sequences. The designation "S1" refers to the following mutations: D189S, Q192M, I138T, insert T219. L1, L2, L40, and L60 refer to the substitution of the designated regions in rat trypsin II with the regions of chymotrypsin.

discrimination between different residues in P1'–P3' usually ranges around 10³-fold (Schellenberger et al., 1993).

Trypsin and chymotrypsin exhibit markedly different preferences for P1' residues (Schellenberger et al., 1994). Trypsin prefers large hydrophobic residues (Met, Ile), while chymotrypsin favors positively charged residues in the P1' position. X-ray crystal structures of different trypsin- and chymotrypsin-inhibitor complexes reveal strong similarities in the backbone conformation of P1'–P3' inhibitor residues (Bode & Huber, 1992). A hydrogen bond is formed between the carbonyl oxygen of Phe 41 and the amide group of the P2' residue and is probably the most important backbone–backbone interaction in the S' subsites in these complexes. As a consequence of the extended backbone conformation of the peptide chain, the P1' and P3' residues point in the same direction and in the opposite direction to P2' residue (Figure 1). Moreover, acyl-transfer experiments indicate that large residues in P1' and P3' compete for similar regions of the enzyme surface. Therefore, the S1' and S3' sites overlap and the specificities are similar (Schellenberger et al., 1994). The structures of rat trypsin complexed with BPTI (Perona et al., 1993) and bovine α -chymotrypsin with OMTKY (Fujinaga et al., 1987) suggest that two surface loops comprise the S1' and S3' sites, residues 34–41 (loop 40) and 58–64 (loop 60). These loops differ markedly in the number and character of residues in trypsin and chymotrypsin (Figures 2 and 3), which could explain the differences in their S1' specificity. Chymotrypsin's preference for P1' and P3' Arg/Lys is attributed to electrostatic interactions with Asp 35 and Asp 64. Consistently, Asp 64 forms a water mediated salt bridge with P3' Arg in the chymotrypsin-OMTKY complex. In contrast, trypsin contains no negatively charged residues in either loop 40 or loop 60. Trypsin's preference for hydrophobic residues in P1' is probably the result of the hydrophobic character of the S1' site. X-ray structures of other serine protease inhibitor complexes suggest that loop 40 and loop 60 are general determinants of S'–P' interactions. These surface loops also contact the P1'–P3' residues in the kallikrein-BPTI, elastase-OMTKY, and thrombin-hirulog 3 complexes (Chen & Bode, 1983; Bode et al., 1992; Qui et al., 1992).

Trypsin and chymotrypsin also differ in their S2' specificity. Interestingly, trypsin prefers positively charged P2' residues while chymotrypsin prefers hydrophobic P2' residues. The structural determinants of S2' specificity are located in analogous regions of both enzymes. In the rat trypsin–BPTI complex, Glu 151 forms a salt bridge with

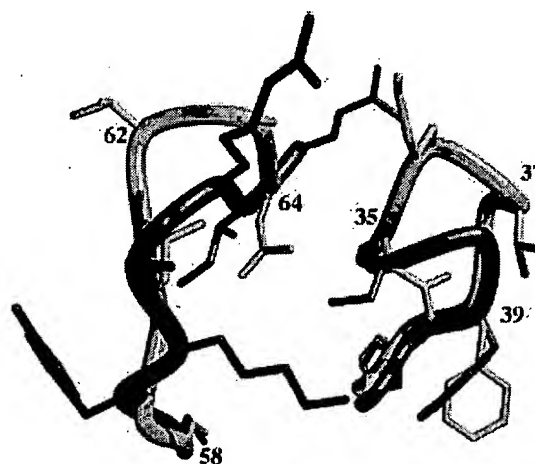


FIGURE 3: The S1' sites of trypsin and chymotrypsin. The structures of rat trypsin II (dark model, from PDB file 1trm) and bovine chymotrypsin (light model, from PDB file 2gch) are shown. The side chains of residues 35–41 and 58–64 are depicted in sticks, while the main chain is depicted as tubes. This figure was created with RAYSCRIPT (obtained from E. Fonatano, D. Peisach, and E. Peisach, Brandeis University), a program that uses the input of MOLSCRIPT (Kraulis, 1991) to generate input for RAYSHADE (Version 4.0, written by C. Kolb and R. Bogart, Princeton University).

P2' Arg; Asn 143 also contacts P2' Arg. In chymotrypsin, residue 151 is Thr, while 143 is Leu, thereby accounting for chymotrypsin's preference for hydrophobic P2' residues.

Few studies have addressed the structural basis of S' specificity. Recent site directed mutagenesis of thrombin identified residues in loop 40 and loop 60 as structural determinants of S2' and S3' specificity (LeBonniec et al., 1996). Site directed mutagenesis of carboxypeptidase Y resulted in an altered S1' specificity (Stennicke et al., 1994). The introduction of negatively charged residues into the S1' site of carboxypeptidase Y dramatically decreased specificity for P1' Phe; however, only a modest increase in preference for P1' Lys was observed with an overall discrimination between Lys and Phe of 1.5-fold. In trypsin, S2' specificity was changed by creating a metal binding site with the mutation of both Asn 143 and Glu 151 to His. Substrate P2' His can also coordinate to the metal, thus introducing His specificity in S2' (Willett et al., 1996). His specificity has also been engineered into the S1' site of subtilisin and trypsin through substrate-assisted catalysis (Carter & Wells, 1987; Corey et al., 1995). Thus, little is known about the structural features which control S' specificity.

In this study we describe the engineering of chymotrypsin-like S1' specificity into the rat trypsin II framework. Loops 40 and 60 were replaced with their chymotrypsin counterparts. Together these substitutions cause a shift of S1' specificity from Met/Ile to Arg/Lys P1' residues.

MATERIALS AND METHODS

Materials. Suc-Ala-Ala-Pro-Phe-AMC, D-Val-Leu-Arg-AMC, and D-Val-Leu-Lys-AMC were purchased from Enzyme Systems Products (Livermore CA). Amino acid amides, dipeptides, dipeptide amides, Z-Arg-AMC, Bz-Arg-OEt, Ac-Phe-OMe, Tos-Gly-Pro-Arg-AMC, and Tos-Gly-Pro-Lys-AMC were purchased from Bachem. H-Trp-Ala-Ala-Ala-Gly-OH was synthesized according to Ullmann and Jakubke (1994). Rat chymotrypsin was a generous gift of Dr. Laszlo Szilagyi (Eötvös Lorand University, Budapest). If not otherwise stated, all chemicals were of analytical grade.

Construction of Trypsin Mutants. Site-directed mutagenesis was performed by the method of Kunkel (1985) as described previously (Hedstrom et al., 1992). All mutants were completely sequenced in order to ensure that only the desired mutations were introduced. The following oligonucleotides were used (mismatches are underlined):

- (1) L40: GTC TCC CTG
CAA GAC TCT ACT GGC TTC CAC TTC TGT
- (2) L60: GCA GCT CAC TGC GGT AAG ACC TCC
GAT GTT GTG GTC GCC GGA GAG CAC AAC

Trypsin and trypsin mutants were isolated and purified as described previously (Hedstrom et al., 1994).

CD Spectroscopy. Samples contained 0.25 mg/mL enzyme in 50 mM Na-acetate buffer, pH 4. Activity was measured before and after CD analysis in order to ensure that no degradation of the enzymes occurred. Spectra were measured at the far-UV range (190–250 nm) using a 1 cm path length cell with a sensitivity of 5×10^{-6} , response time of 2 s at 0.2 nm intervals, and 1 cycle on a Jovin-Yvon Mark V autodichrograph.

Activity of Mutant Trypsins. Assay mix contained 50 mM Hepes, pH 8, 10 mM CaCl_2 , and 0.1 M NaCl. Stock solutions of AMC substrates were prepared in dimethylformamide. The final concentration of dimethylformamide in the assay solution was less than 5%. Hydrolysis of the AMC substrates was monitored fluorometrically, with excitation wavelength of 380 nm and emission wavelength at 460 nm (Zimmerman et al., 1977). Assays were performed using 0.2 mL of assay mix containing substrates (usually 0.5 μM to 2 mM) in a PerSeptive Cyto Fluor spectrofluorimeter at 25 °C. Concentrations of fluorimetric substrates were determined by using a standard curve of AMC fluorescence at 15 different AMC concentrations (0.2–200 μM). Data were analyzed using KinetAsyst software, and reported values are the average of at least two experiments.

Acyl-Transfer Experiments. Reactions were performed at 25°C. A 4 mM stock solution of the acyl donors Bz-Arg-OEt and Ac-Phe-OMe was prepared daily in water. The Ac-Phe-OMe stock contained 1% dimethylformamide. Stock solutions of the amino acid amides (30 mM) were prepared in assay mix readjusted with NaOH to pH 8. The total assay volume was 65 μL . The final acyl donor concentration was

2 mM and the nucleophile concentrations 15 mM, calculated as unprotonated amino acid amide concentration $[\text{N}]$ according to the following equation ($[\text{NH}]_0$ corresponds to the total nucleophile concentration):

$$[\text{N}] = [\text{NH}]_0 / (1 + 10^{\text{pK} - \text{pH}}) \quad (1)$$

The acyl-transfer reaction was initiated with 5 μL of enzyme stock solution. Enzyme concentration and reaction time were adjusted to achieve an ester consumption of 50–80% in order to ensure that no secondary hydrolysis of the formed peptide product occurred. The reaction was stopped by diluting 50 μL of the reaction mixture in 0.3 mL 50% aqueous methanol and 1% trifluoroacetic acid. The partition values were determined from three to five independent experiments. Control experiments without the enzyme were performed in order to estimate non enzymatic ester hydrolysis (0–1.5%). HPLC was performed using a Hewlett-Packard 1090LC system (Palo Alto, CA) on a Vydac analytical reversed phase C_{18} column (Vydac 218TP.54). Samples were eluted under isocratic conditions with eluents containing 15–25% acetonitrile (depending on the nucleophile) in 0.1% aqueous trifluoroacetic acid at flow rates of 1.0–1.2 mL min^{-1} . Absorbance was monitored at 254 nm (Bz-Arg-OEt) and 220 nm (Ac-Phe-OMe). The ratio between aminolysis and hydrolysis product was calculated from the corresponding peak areas. The ratio of extinction coefficients of the sample components was obtained by hydrolysis experiments according to Ullmann et al., (1994).

RESULTS AND DISCUSSION

Design of Mutant Trypsins. We constructed four mutant trypsins in order to probe the role of loop 40 and loop 60 in determining the S1' specificity of trypsin and chymotrypsin (Figure 2). $\text{Tr}' \rightarrow \text{Ch}'[\text{L40}]$ and $\text{Tr}' \rightarrow \text{Ch}'[\text{L60}]$ contain the amino acid sequence of chymotrypsin in positions 34–39 and 59–68, respectively (chymotrypsinogen numbering). $\text{Tr}' \rightarrow \text{Ch}'[\text{L40+L60}]$ combines both mutations. In addition, a fourth mutant was constructed from the chymotrypsin-like mutant $\text{Tr} \rightarrow \text{Ch}[\text{S1+L1+L2+Y172W}]$ in order to probe the interdependence of S1 and S1' specificity (Hedstrom et al., 1994). This mutant, $\text{Tr} \rightarrow \text{Ch}[\text{S1+L1+L2+Y172W+L40+L60}]$, also contains both loop 40 and loop 60. All four mutant trypsins are expressed in a *Saccharomyces cerevisiae* system (Hedstrom et al., 1994). They were purified using standard methods and remained stable in 1 mM HCl.

The Secondary Structures of $\text{Tr}' \rightarrow \text{Ch}'[\text{L40+L60}]$ and $\text{Tr} \rightarrow \text{Ch}[\text{S1+L1+L2+Y172W+L40+L60}]$ Differ from Trypsin. Figure 4 shows the CD spectra of $\text{Tr}' \rightarrow \text{Ch}'[\text{L40+L60}]$, $\text{Tr} \rightarrow \text{Ch}[\text{S1+L1+L2+Y172W+L40+L60}]$, trypsin, and bovine chymotrypsin. The spectra of the mutant enzymes display substantial changes in the 200–220 nm range. The CD spectrum in this wavelength range is determined by the secondary structure of the protein. The minimum of the spectrum of trypsin is 211 nm while that of chymotrypsin is 204 nm. Interestingly, the minimum of the spectra of the mutant enzyme is shifted to lower wavelengths, which may suggest that the structure of these enzymes is more chymotrypsin-like.

Activity of Mutant Trypsins. The hydrolysis of amide substrates by the mutant trypsins was characterized in order to assess the influence of loop 40 and loop 60 on S1

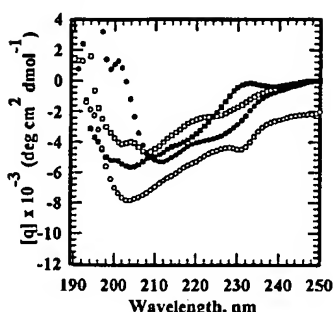


FIGURE 4: CD spectra of trypsin, chymotrypsin, and mutant enzymes. Samples contained 0.25 mg/mL enzyme in 50 mM Na-acetate buffer pH 4. Spectra were measured at the far UV range (190–250 nm) using a 1 cm path length cell with a sensitivity of 5×10^{-6} , response time of 2 s at 0.2 nm intervals, and 1 cycle on a Jovin–Yvon Mark V autodichrograph. Closed circles, rat trypsin S195A; open circles, bovine chymotrypsin; closed boxes, Tr→Ch[L40+L60]; open boxes Tr→Ch[S1+L1+L2+Y172W+L40+L60].

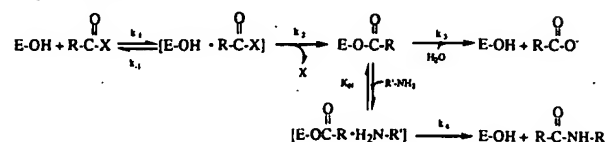
Table 1: Steady-State Kinetic Parameters for the Hydrolysis of Substrates by Trypsin and Trypsin Mutants^a

substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
rat trypsin ^b			
Tos-Gly-Pro-Arg-AMC	3.5	29	9.0×10^6
Tos-Gly-Pro-Lys-AMC	12	17	1.4×10^6
D-Val-Leu-Arg-AMC	39	17	4.2×10^5
D-Val-Leu-Lys-AMC	395	13	3.1×10^4
Z-Arg-AMC	55	0.5	9.6×10^3
Tr→Ch[L40]			
Tos-Gly-Pro-Arg-AMC	2	11	8.0×10^6
Tos-Gly-Pro-Lys-AMC	18	28	1.5×10^6
D-Val-Leu-Arg-AMC	13	4	3.6×10^5
D-Val-Leu-Lys-AMC	106	6	5.3×10^4
Z-Arg-AMC	79	0.6	7.0×10^3
Tr→Ch[L60]			
Tos-Gly-Pro-Arg-AMC	5	31	6.3×10^6
Tos-Gly-Pro-Lys-AMC	15	19	1.3×10^6
D-Val-Leu-Arg-AMC	19	7	3.7×10^5
D-Val-Leu-Lys-AMC	215	6	2.7×10^4
Z-Arg-AMC	31	0.3	8.5×10^3
Tr→Ch[L40]+L60]			
Tos-Gly-Pro-Arg-AMC	4	9	2.0×10^6
Tos-Gly-Pro-Lys-AMC	18	10	7.5×10^5
D-Val-Leu-Arg-AMC	45	2	7.8×10^4
D-Val-Leu-Lys-AMC	66	2	3.7×10^4
Z-Arg-AMC	58	0.3	5.2×10^3
Tr→Ch [S1+L1+L2+Y172W+L40+L60]			
Suc-AlaAlaProPhe-AMC	$\geq 3000^c$	≥ 30	9.0×10^3
Tr→Ch [S1+L1+L2+Y172W] ^d			
Suc-AlaAlaProPhe-AMC	≥ 3000	≥ 30	9.3×10^3

^a Conditions: 50 mM Hepes, pH 8, 100 mM NaCl, 10 mM CaCl₂, 30 °C, λ_{ex} = 380 nm, λ_{em} = 460 nm. ^b Hedstrom et al.; 1994. ^c No saturation with 1 mM substrate, 37 °C; all errors are less than 15%. ^d Hedstrom et al., 1996.

specificity. Tr→Ch[L40], Tr→Ch[L60], and Tr→Ch[L40+L60] are very similar to trypsin as shown by the Michaelis–Menten parameters in Table 1. Like trypsin, the mutant enzymes hydrolyze oligopeptide substrates much more efficiently than single amino acid substrates (by a factor of 10^3 as measured by k_{cat}/K_m). This observation underlines the role of S2–S4 subsites for specific and efficient substrate processing. As expected, the mutations in loop 40 and loop 60 do not influence the primary specificity and enzymatic activity of trypsin. Similarly, the chymotrypsin-like mutant Tr→Ch[S1+L1+L2+Y172W+L40+L60] exhibits an iden-

Scheme 1: Mechanism for Serine Protease Catalyzed Acyl-Transfer Reactions^a



^a Enzyme is denoted E-OH, acyl donor is R-COX, where X is the leaving group, R'-NH₂ is the nucleophile.

tical value of k_{cat}/K_m for the hydrolysis of Suc-Ala-Ala-Pro-Phe-AMC as Tr→Ch[S1+L1+L2+Y172W].

Determination of S1' Specificity. S1' specificity can be determined by monitoring acyl-transfer reactions with added nucleophiles. This reaction is the reverse of peptide hydrolysis and therefore provides analogous specificity data (Scheme 1; Fersht et al., 1973; Schellenberger & Jakubke, 1991; Schellenberger et al., 1993). This postulate has been validated for a number of serine and cysteine proteases (Stein et al., 1987; Schellenberger et al., 1994; Ullmann & Jakubke, 1994). Since the acyl group of a substrate (R-COX, which is usually an ester) can be transferred both to water and R'-NH₂, two products are formed: R-CO₂H and R-CO-NHR', respectively. The ratio between hydrolysis and aminolysis is determined by the S' specificity of the protease. The partition value p describes acyl-transfer efficiency and therefore the S1' specificity of a protease. p is defined according to Scheme 1 as

$$p = [\text{R}'\text{-NH}_2](v_H/v_A) = k_3K_N/k_4 \quad (2)$$

where v_H and v_A represent the velocities of hydrolysis and aminolysis, respectively. The partition value can be determined from the product ratios when R'-NH₂ is in excess:

$$p = [\text{R}'\text{-NH}_2]_0[\text{R-CO}_2\text{H}]/[\text{R-CONH-R'}] \quad (3)$$

where $[\text{R}'\text{-NH}_2]_0$ is the initial nucleophile concentration and $[\text{R-CO}_2\text{H}]$ and $[\text{R-CONH-R'}]$ represent the product concentrations. In this work, we will utilize the parameter $1/p$ in order to emphasize the aminolysis reaction. Consequently, an increase in the value $1/p$ can be directly correlated with the preference of the protease for a given R'-NH₂. Therefore, the S1' specificity of trypsin and the mutant enzymes was determined by measuring $1/p$ for 16 amino acid amide nucleophiles, using Bz-Arg-OEt and Ac-Phe-OMe as the acyl donors as appropriate.

S1' Specificity of Trypsin, Chymotrypsin, and Tr→Ch[S1+L1+L2+Y172W]. Figure 5, panels a and b, shows the $1/p$ values for the trypsin and chymotrypsin-catalyzed acyl-transfer reactions. These values are consistent with literature data (Fersht et al., 1973; Schellenberger & Jakubke, 1991; Gololobov et al., 1992). In chymotrypsin-catalyzed reactions, $1/p$ for Arg-NH₂ is 3–5-fold higher than $1/p$ for aromatic amino acid amides and 8-fold higher than $1/p$ for aliphatic amino acid amides, while the specificity for small and negatively charged amino acid amides is 50–500-fold lower. This result does not agree with previous observations of Schellenberger et al. (1993), who measured acyl-transfer to pentapeptide nucleophiles and observed a much lower preference for aromatic P1' residues. Therefore, we performed acyl-transfer reactions to H-Trp-Ala-Ala-Ala-Gly-OH with chymotrypsin. $1/p$ decreases 6-fold for H-Trp-Ala-Ala-Ala-Gly-OH compared to $1/p$ for H-Trp-NH₂ (data not

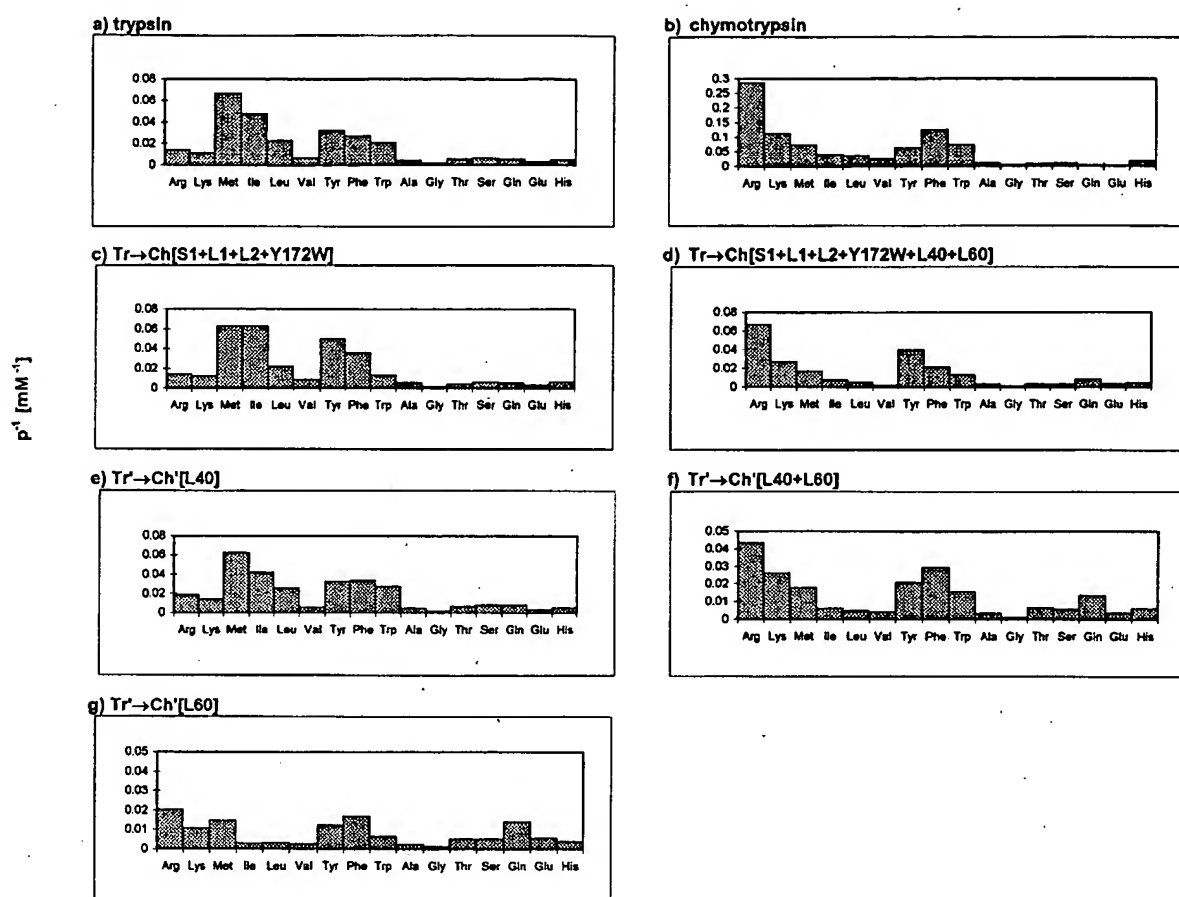


FIGURE 5: S_1' specificity of trypsin, chymotrypsin and mutant enzymes. Assays were performed at 25 °C in assay mix (50 mM Hepes, pH 8, 10 mM CaCl_2 , 100 mM NaCl, 0.01% Triton) containing 2 mM Bz-Arg-OEt or Ac-Phe-OMe and 15 mM R-NH₂. Reactions were started with enzyme. Reactions were stopped by the addition of 50% methanol and 1% TFA after 50–80% of the ester was consumed. Products were analyzed via reversed phase HPLC (Vydac CAT 218TP54, 12–20% acetonitrile, $\lambda = 254$ and 220 nm). The partition value was calculated as $p = [\text{Ac-OH}][\text{R-NH}_2]/[\text{Ac-NH-R}]$. Since $[\text{R-NH}_2] \gg [\text{Bz-Arg-OEt}]$ and Ac-Phe-OMe, it can be assumed that $[\text{R-NH}_2]$ remains constant during the course of reaction. (a) Wild-type rat trypsin. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0015. (b) Rat chymotrypsin. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0032, for acyltransfer to Gln-NH₂ is 0.0032, and for acyltransfer to Glu-NH₂ is 0.0010. (c) Tr \rightarrow Ch[S1+L1+L2+Y172W]. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0018. (d) Tr \rightarrow Ch[S1+L1+L2+Y172W+L40+L60]. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0009. (e) Tr' \rightarrow Ch'[L40]. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0017. (f) Tr' \rightarrow Ch'[L40+L60]. The value of $1/p$ for acyltransfer to Gly-NH₂ is 0.0012.

shown). This observation implies that P_1' aromatic residues have different binding modes in oligopeptide nucleophiles and amino acid amide nucleophiles in chymotrypsin.

In trypsin-catalyzed acyl-transfer reactions, the values of $1/p$ for Met-NH₂ and Ile-NH₂ are 1.5–3-fold higher than for Phe-NH₂ and 5-fold higher than for Arg-NH₂. Again, the specificity for small and negatively charged amino acid amides is low, although the specificity for negatively charged residues is much higher than in chymotrypsin. These data are consistent with the values for the analogous pentapeptide nucleophiles (Schellenberger et al., 1993).

S_1' Specificity of Mutant Trypsins. Surprisingly, the exchange of the loop 40 in trypsin has only a modest effect on the S_1' specificity of trypsin. The specificity for positively charged residues in P_1' is only minimally increased and the S_1' specificity for all other nucleophiles remains unchanged (Figure 5e). This observation suggests that loop 40 does not influence the S_1' specificity.

Figure 5g shows that the substitution of loop 60 in trypsin causes a 5–15-fold decrease in specificity for aliphatic amino acid amides. While the preference for most nucleophiles remained constant or dropped, moderate increases in the

values of $1/p$ for Arg-NH₂ and Gln-NH₂ are observed. Consequently, Tr' \rightarrow Ch'[L60] prefers P_1' Arg and Phe. Nevertheless, the replacement of loop 60 with the analogous chymotrypsin sequence does not create a strong preference for Arg-NH₂ as observed in chymotrypsin.

While alone loop 40 and loop 60 fail to reconstitute chymotrypsin-like S_1' specificity, the mutant combining substitutions in both loops, Tr' \rightarrow Ch'[L40+L60], has a dramatically changed S_1' specificity with a strong preference for P_1' Arg. Figure 5f shows that $1/p$ for acyl-transfer to Arg-NH₂ is increased 3-fold relative to trypsin. The specificity for Lys-NH₂ is 2.5-fold higher than in trypsin. In contrast, the preference for Leu-NH₂, Ile-NH₂, and Met-NH₂ is decreased 4–8-fold. Therefore, the exchange of both loop 40 and loop 60 increases the relative P_1' Arg/Met discrimination of trypsin by factor 12 and the P_1' Arg/Ile preference by factor 24. The specificity for the other nucleophiles is similar to trypsin. Surprisingly, Tr' \rightarrow Ch'[L40+L60] retains trypsin-like specificity for negatively charged nucleophiles despite the introduction of two Asp residues. Overall the values of $1/p$ for Tr' \rightarrow Ch'[L40+L60] remain about 7-fold lower than those observed for chymo-

trypsin-catalyzed acyl-transfer reactions. Nevertheless, the ratio between the values of $1/p$ for Arg-NH₂ and Met-NH₂ are similar for Tr \rightarrow Ch[L40+L60] and chymotrypsin. Thus, our data demonstrate that loop 40 and loop 60 work synergistically to determine the chymotrypsin-like S1' specificity.

Interdependence of S1 and S1' subsite. Table 1 and Figure 5 reveal that dramatic changes in the character of the S' subsite can be introduced without significant influence on the primary specificity of the enzyme. Acyl-transfer experiments were performed with Tr \rightarrow Ch[S1+L1+L2+Y172W] and Tr \rightarrow Ch[S1+L1+L2+Y172W+L40+L60] in order to further assess the influence of the S1 subsite on S1' specificity. No differences in S1' specificity of Tr \rightarrow Ch'-[S1+L1+L2+Y172W] and trypsin are observed as measured by acyl-transfer to amino acid amides as nucleophiles (Figure 5, panels a and c). This result is in apparent conflict with Schellenberger et al. (1993), who observed a significant decrease in specificity for P1' Phe/Tyr/Trp containing pentapeptide nucleophiles in the chymotrypsin-like mutant Tr \rightarrow Ch'[S1+L1+L2] compared to trypsin. Therefore, we performed acyl-transfer reactions to H-Trp-Ala-Ala-Ala-Gly-OH with Tr \rightarrow Ch[S1+L1+L2+Y172W]. $1/p$ decreases 4-fold for H-Trp-Ala-Ala-Ala-Gly-OH compared to H-Trp-NH₂ (data not shown). As discussed above, similar differences in acyl-transfer to P1' aromatic amino acid amides and oligopeptides have been observed in chymotrypsin. In contrast, no significant difference in specificity for H-Trp-Ala-Ala-Ala-Gly-OH and H-Trp-NH₂ was obtained for trypsin and Tr \rightarrow Ch[L40+L60]. Thus, P1' aromatic residues have different binding modes in oligopeptide and amino acid amide nucleophiles in chymotrypsin-like enzymes, but not in trypsin-like enzymes. This observation indicates that the S1 subsite can have modest influence on S1' specificity.

Figure 5d shows the acyl-transfer data for Tr \rightarrow Ch-[S1+L1+L2+Y172W+L40+L60] with Ac-Phe-OMe as the acyl donor. Again the simultaneous substitution of loop 40 and loop 60 strongly affects the S1' specificity. Since $1/p$ for Arg-NH₂ increases 5-fold while Met-NH₂ and Ile-NH₂ drop 4–8-fold, the P1' Arg/Met and Arg/Ile discrimination changed 20–40-fold relative to the parent enzyme. As in Tr \rightarrow Ch[L40+L60], no change is observed in $1/p$ for Phe-NH₂, Tyr-NH₂, and Trp-NH₂ compared to the parent enzyme. The values of $1/p$ for small and negatively charged residues are comparable to Tr \rightarrow Ch[S1+L1+L2+Y172W]. Tr \rightarrow Ch[S1+L1+L2+Y172W+L40+L60] has a 1.6 fold higher P1' Arg preference than Tr \rightarrow Ch[L40+L60]. This observation further indicates that the S1 site can have a modest effect on S1' specificity.

The Mutation of Loop 40 and Loop 60 Causes a Change in the Kinetic Course of the Acyl-Transfer Reaction. Scheme 1 requires that the product ratio $[R\text{-CONH-R}']/[R\text{-CO}_2\text{H}]$ is linearly dependent on the R'-NH₂ concentration. This dependence is observed for trypsin-catalyzed acyl-transfer to Arg-NH₂, as well as for Tr \rightarrow Ch[S1+L1+L2+Y172W]-catalyzed acyl-transfer to Arg-NH₂ (Figure 6). However, data for Tr \rightarrow Ch[L40+L60] and Tr \rightarrow Ch-[S1+L1+L2+Y172W+L40+L60]-catalyzed acyl-transfer to Arg-NH₂ do not fit this model. Figure 6 shows $[R\text{-CONH-R}']/[R\text{-CO}_2\text{H}]$ is not linearly dependent on R'-NH₂ for both of these mutant enzymes. This deviation occurs at much higher nucleophile concentrations than those used in the previous experiments, and thus does not change the conclusions based on Figure 5. This deviation may result from a

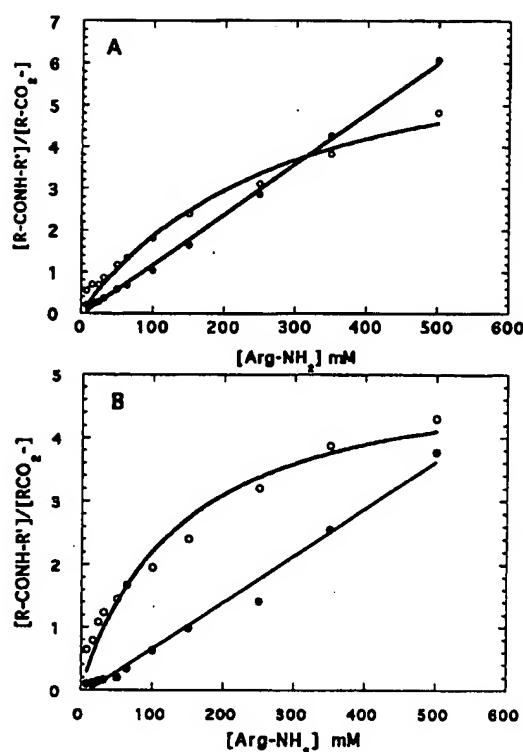
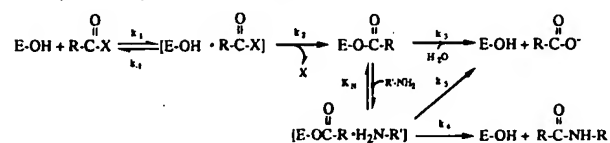


FIGURE 6: Dependence of $[R\text{-CONH-R}']/[R\text{-COO}^-]$ on $[R'\text{-NH}_2]$. Conditions as described in Figure 5. (a) Open circles, Tr \rightarrow Ch[L40+L60], closed circles, rat trypsin. (b) Open circles, Tr \rightarrow Ch[S1+L1+L2+Y172W+L40+L60]; closed circles, Tr \rightarrow Ch[S1+L1+L2+Y172W].

Scheme 2: Alternative Mechanism of Serine Protease Catalyzed Acyl-Transfer Reactions^a



^a Enzyme is denoted E-OH, acyl donor is R-COX, where X is the leaving group, R'-NH₂ is the nucleophile.

change in ionic strength or other solvent properties due to high R'-NH₂ concentrations. However, we do not favor this explanation because this effect is not observed in the parent enzymes. Alternatively, this deviation is consistent with the model shown in Scheme 2, where the acyl enzyme-nucleophile complex can undergo both hydrolysis and aminolysis (Schellenberger & Jakubke, 1991; Gololobov et al., 1993). In this model, $[R\text{-CONH-R}']/[R\text{-CO}_2\text{H}] = k_4/k_5$ at infinite R'-NH₂ concentrations. Thus the mutations in loop 40 and loop 60 appear to change the kinetic course of the acyl-transfer reaction. The hydrolysis of the acyl enzyme-nucleophile complex indicates that R'-NH₂ binds nonproductively to the acyl enzyme. This nonproductive binding mode suggests that the new S1' sites are defective.

Summary. This work demonstrates that the S1' specificity of trypsin and chymotrypsin is synergistically determined by two surface loops, loop 40 and loop 60. However, neither Tr \rightarrow Ch[L40+L60] nor Tr \rightarrow Ch[S1+L1+L2+Y172W+L40+L60] are equivalent to chymotrypsin in the acyl-transfer efficiency, which indicates that additional structural determinants of the S1' specificity in trypsin and chymotrypsin remain to be identified.

ACKNOWLEDGMENT

The authors thank Rebecca Myers of the Brandeis DNA Facility for DNA sequencing and oligonucleotide preparation, and Eben Kunz for help with graphics.

REFERENCES

- Bastos, M., Maeji, N. J., & Abeles, R. H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 6738–6742.
- Bode, W., & Schwager, P. (1975) *J. Mol. Biol.* 98, 693–717.
- Bode, W., & Huber, R. (1992) *Eur. J. Biochem.* 204, 433–451.
- Bode, W., Turk, D., & Karshikov, A. (1992) *Protein Sci.* 1, 426–471.
- Carter, P., & Wells, J. A. (1991) *Science* 237, 394–399.
- Chen, Z., & Bode, W. (1983) *J. Mol. Biol.* 164, 283–311.
- Corey, D. R., Willett, W. S., Coombs, G. S., & Craik, C. S. (1995) *Biochemistry* 34, 11522–11527.
- Fersht, A. R., Blow, D. M., & Fastrz, J. (1973) *Biochemistry* 12, 2035–2041.
- Fujinaga, M., Sielecki, A. R., Read, R. J., Ardelt, W., Laskowski, M., Jr., & James, M. N. G. (1987) *J. Mol. Biol.* 195, 397–418.
- Gololobov, M. Y., Voyushina, T., Stepanov, V., & Adlercreutz, P. (1993) *Eur. J. Biochem.* 217, 955–963.
- Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) *Science* 255, 1249–1253.
- Hedstrom, L., Perona, J., & Rutter, W. J. (1994) *Biochemistry* 33, 8757–8763.
- Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol.* 89, 73–101.
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* 24, 946–950.
- Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 488–492.
- Le Bonniec, B. L., Myles, T., Johnson, T. Knight, C. G., Tapparelli, C., & Stone, S. R. (1996) *Biochemistry* 35, 7114–7122.
- Perona, J. J., Hedstrom, L., Rutter, W. J., & Fletterick, R. J. (1995) *Biochemistry* 34, 1489–1499.
- Perona, J. J., Hsu, C. A., Craik, C. S., & Fletterick, F. J. (1993) *J. Mol. Biol.* 230, 919–933.
- Polgar, L. (1989) *Mechanisms of Protease Action*, CRC Press, Inc., Boca Raton.
- Qui, X., Padmanabhan, K. P., Carperos, V. E., Tulinski, A., Kline, T., Maraganore, J. M., & Fenton, J. W. (1992) *Biochemistry* 31, 11689–11697.
- Remington, S. J., Woodbury, R. G., Reynolds, R. A., Matthews B. W., & Neurath, H. (1988) *Biochemistry* 27, 8097–8105.
- Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162.
- Schellenberger, V., & Jakubke, H.-D. (1991) *Angew. Chem., Int. Ed. Engl.* 30, 1437–1449.
- Schellenberger, V., Turck, C. W., Hedstrom, L., & Rutter, W. J. (1993) *Biochemistry* 32, 4349–4353.
- Schellenberger, V., Turck, C. W., & Rutter, W. J. (1994) *Biochemistry* 33, 4251–4257.
- Stein, R. L., & Strimpler, A. M., (1987) *Biochemistry* 26, 2238–2242.
- Stennicke, H. R., Ujje, H. M., Christensen, U., Remington, S. J., & Breddam, (1994) *Protein Eng.* 7, 911–916.
- Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol.* 83, 185–208.
- Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry* 13, 4212–4228.
- Ullmann, D., & Jakubke, H. D. (1994) *Eur. J. Biochem.* 223, 865–872.
- Willett, W. S., Brinen, L. S., Fletterick, R. J., Craik, C. S. (1996) *Biochemistry* 35, 5992–5998.
- Zimmerman, M., Ashe, B., Yurewicz, E. C., & Patel, G. (1977) *Anal. Biochem.* 78, 47–51.

BI970937L